Brain Research, 404 (1987) 58–64
Elsevier

BRE 12351

Selective release of endogenous zinc from the hippocampal mossy fibers in situ

L. Anikszteln, G. Charton and Y. Ben-Ari

INSERM Unité 029, Hôpital de Port-Royal, Paris (France) and C.N.R.S., Gif-sur-Yvette (France)

(Accepted 1 July 1986)

Key words: Hippocampus; In situ; Push–pull zinc; Mossy fiber

The release of endogenous zinc was studied in the hippocampus of the anesthetized rat. Push–pull cannulae were bilaterally introduced in the hippocampus and zinc concentrations determined by atomic absorption spectrophotometry. We first studied the regional distribution of K^+-evoked release of zinc. A 2 min (30 mM) K^+ pulse produced a release of endogenous zinc, when the push–pull cannulae were located in the vicinity of the mossy fibers (CA3 or hilus) but not in other regions (including CA1, fimbria, molecular layer of the fascia dentata, thalamus etc...). In CA3 the maximal release was of 2000 ng/ml (200 times the levels of zinc present in the cerebrospinal fluid (CSF)). Destruction of the mossy fibers by a local unilateral injection of 1.5 μg of colchicine dissolved in 0.6 μl of a saline solution, eliminated this release without affecting the release in the control (contralateral) side. Electrical stimulation of the perforant path at 1 Hz did not evoke a release of zinc. In contrast at 10 Hz this stimulation produced a burst of population spike and a significant release of zinc in the mossy fibers (and not in other regions of the hippocampus). These experiments provide direct evidence that zinc is selectively released from the mossy fibers.

INTRODUCTION

Zinc plays an important role in a large number of biological functions[10,11], and there has been considerable interest concerning the role of this metal in neurobiology[14]. Following the initial histochemical observations by Maske[25], a large number of studies have shown that in human and rat brains the hippocampal mossy fibers contain particularly high concentrations of zinc[9,15,22]. In addition to qualitative histochemical methods relying on the Timm stain[18a] or quantitative histochemistry[12,13], electron microscopic studies indicate that the zinc is present in mossy fiber terminals[18,23]. Furthermore, radioactive zinc is accumulated in the mossy fibers following parenteral administration[30], and an uptake system for radioactive zinc has been described in hippocampal slices[21]. These observations raise the possibility that zinc plays a role in synaptic transmission in the mossy fibers[7,20,30]. In keeping with this, zinc reduces paired pulse potentiation in the hippocampus[24].

To better understand the role of zinc in the mossy fibers, it is essential to determine if it is released following synaptic activation. Recent studies have shown a release of endogenous zinc by a K^+ pulse applied to the hippocampus in situ[6] and to the hippocampal slice preparation[4]. The aim of the present study is to provide direct evidence that zinc is selectively released from the mossy fibers. For this reason we have examined the following in the hippocampus of the anesthetized rat: (a) the regional distribution of the sites from which a K^+ pulse evokes a release of zinc; (b) the effect of prior destruction of the mossy fibers on this release; (c) the release of endogenous zinc produced by electrical stimulation of the perforant path. Some of the present observations have been reported in brief[3,6].

MATERIAL AND METHODS

Forty adult male Wistar rats (200–300 g) were used in the present study. These were anesthetized

Correspondence: L. Anikszteln, INSERM Unité 029, Hôpital de Port-Royal, 123 Bd. de Port-Royal, 75014 Paris, France.

with urethane (Sigma) (2 g/kg), and placed in a stereotaxic frame. A push–pull cannula was introduced in the hippocampus of each hemisphere. The anteriority was always constant (A = 4.2, atlas of Albe-Fessard et al.[1]); the laterality varied between 1.5 and 4. To ascertain the exact position of the cannula, we relied on the typical field potentials produced by electrical stimulation of the perforant path[2]. To achieve this a recording bipolar electrode, which had the same diameter (0.2 mm o.d.) and length as the inner cannula, was introduced inside the outer cannula (0.5 mm o.d.). An electrode was also implanted in the entorhinal cortex (A = 0.2; L = 4.5; H = 6.4) to stimulate the perforant path. Once the cannula was adequately placed, the recording electrode was removed and replaced by the inner cannula. Then the push–pull cannula was provided with artificial cerebrospinal fluid (CSF) with the following composition (mM): 126.5 NaCl; 2.4 KCl; 2 $CaCl_2$; 0.83 $MgCl_2$; 0.5 KH_2PO_4; 0.5 Na_2SO_4; 27.5 $NaHCO_3$; 5.9 glucose;

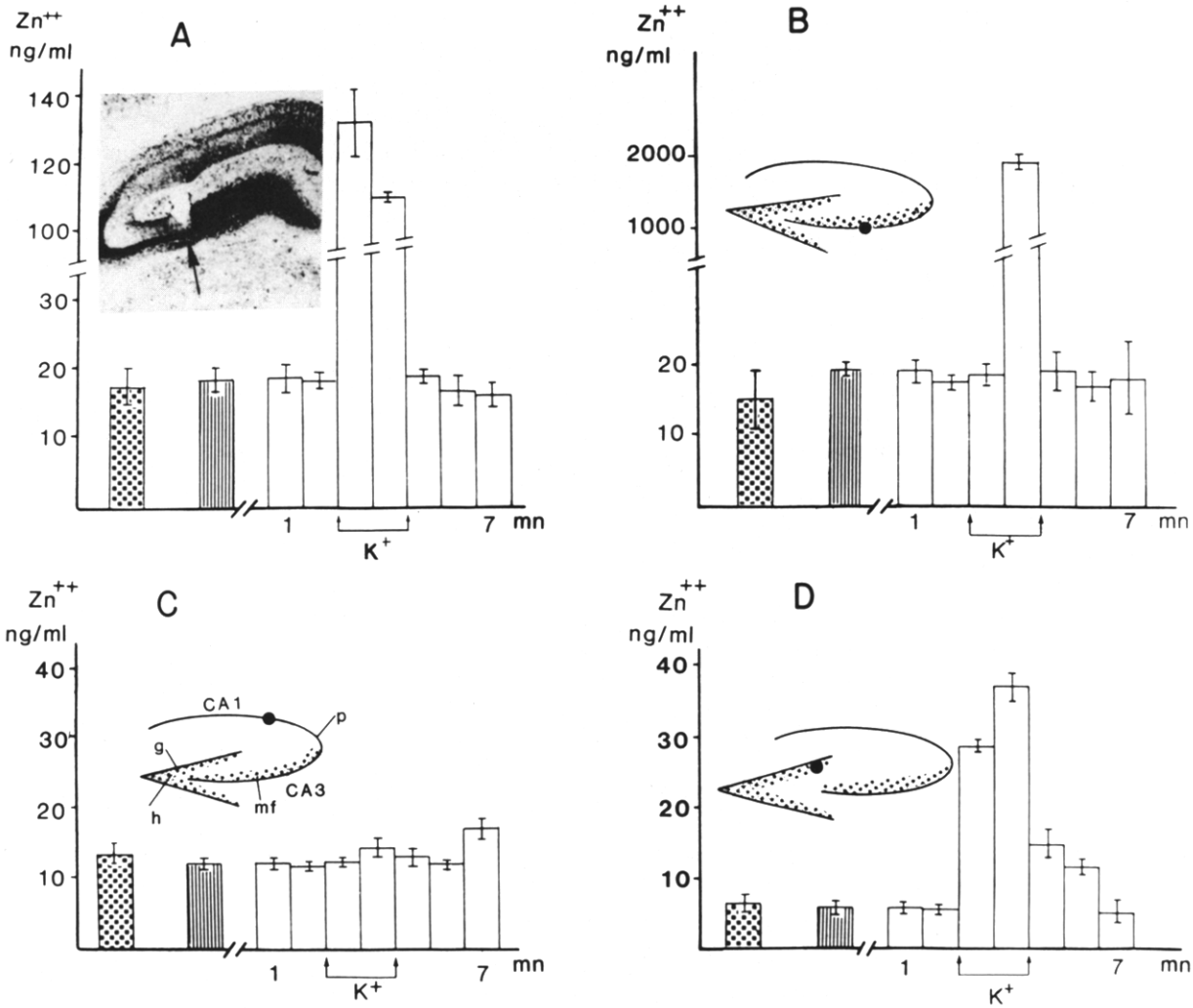

Fig. 1. K^+-evoked release of endogenous zinc. In this and following figures, each clear column represents the mean concentration of zinc found in 1 min samples (11 μl). Each assay was made in triplicate and the standard deviations are indicated (vertical bars). The location of the cannulae (as revealed by histological procedures) is depicted in the schematic diagrams (dark circle). In each experiment, the concentrations of zinc present in the artificial CSF were first measured (spotted column). Once the cannula was placed and after the stabilization period, we determined the concentrations of zinc in 4–6 samples, this was taken as a measure of possible spontaneous release of zinc (hatched column). The K^+ pulse (30 mM, 2 min) produced a release of zinc when the cannula was localized in CA3 (A, B) or in the hilus (D) but not in CA1 (C). Abbreviations: g, granular layer; h, hilus; mf, mossy fibers; p, pyramidal cells.

the pH was adjusted to 7.4, and the flow rate was of 11 μl/min. The perfusion was continued for 1 h to allow stabilization. After the stabilization period, 4–6 samples were collected to determine spontaneous release. A brief pulse of 30 mM K^+ was applied unilaterally during 2 min, and samples collected from both hippocampi for 15 min. The concentration of zinc in each 1 min sample was determined with an atomic absorption spectrophotometer (VARIAN AA 175 with a graphite chamber GIA 175). To minimize trace contamination of zinc the following procedure was used. Every item, including cannulae and catheters, was washed with nitric acid followed by deionized water for 1 h. We used only suprapure chemicals for the preparation of the CSF. The following zinc concentrations were found in the CSF: 13.22 ± 8.73 ng/ml (n = 30 samples); similar values were found when 30 mM K^+ was added: 12.63 ± 7.44 ng/ml (n = 30).

In a second experimental series (n = 6), the mossy fibers were unilaterally destroyed with a local injection of colchicine[17] (two injections of 1.5 μg of colchicine dissolved in 0.6 μl of a saline solution were stereotaxically injected in the septal and temporal poles of the fascia dentata) and the release evoked by a K^+ pulse was studied 6–7 days later. The contralateral hippocampus served as a control.

In a third experimental series, the release of zinc produced by electrical stimulation was studied. For this purpose, one perforant path was stimulated at a frequency of 1 Hz (4 min), or 10 Hz (5 min) (pulses of 0.5 ms; 1–2 V which corresponds to 1.5 times the threshold to elicit population spikes).

At the end of each experiment the animal was perfused intracardially with saline or sulphide solution followed by 4% paraformaldehyde[29]. Conventional histological procedures were performed to determine the position of the cannulae and stimulating electrode. This procedure included Nissl staining and a modified Timm procedure[29] to determine the extent of the destruction of the mossy fibers.

RESULTS

Release of zinc by a K^+ pulse

When the push–pull cannulae were localized in the mossy fiber region of CA3 (a, b or c), (n = 12), a K^+ pulse consistently produced a significant release of endogenous zinc (Figs. 1A, B and 2A). The maximal concentrations of zinc produced in the perfusate by the K^+ pulse was of 2000 ng/ml (200 times the levels found in the control CSF solutions). The latency and duration of this response were brief (usually less than 2 min). In contrast K^+ failed to evoke a release in stratum pyramidale or radiatum of CA1 (n = 9), in the granular and molecular layers of the fascia dentata (n = 7), in the thalamus (n = 2) or in the fimbria (n = 1).

The hilar zone of the fascia dentata gave a more complex picture. K^+ produced a release of zinc in 4 out of 7 cases (Figs. 1D and 2A). This release differed from that observed in CA3 in terms of duration and amplitude. Thus as shown in Fig. 1D, the peak response which was reached progressively was relatively small (3–4-fold the levels of the control CSF) and outlasted the duration of the K^+ pulse. The hilar zone of the fascia dentata also differed from CA3 in

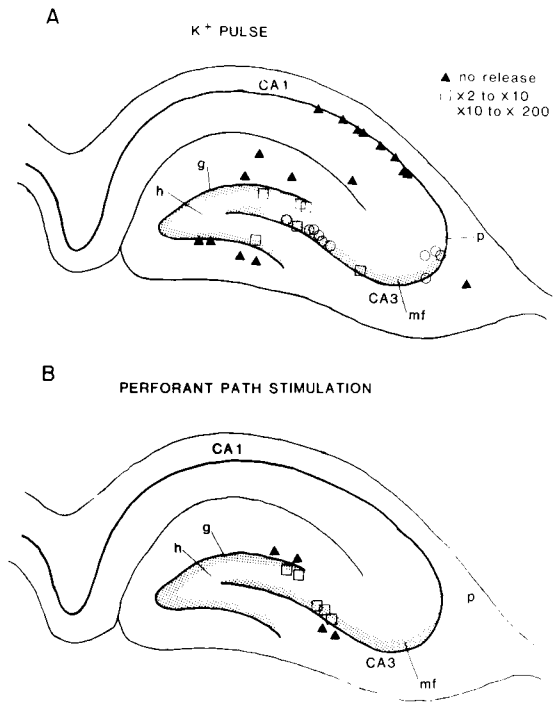

Fig. 2. Regional distribution of the release of endogenous zinc in the hippocampus produced (A) by a K^+ pulse and (B) by the electrical stimulation of the perforant path. The symbols indicate the localization of the push–pull cannulae, and indicate (see legend) the concentrations of zinc released with reference to the artificial CSF. Both procedures evoked a release of zinc in CA3 and in the hilus but not in other regions.

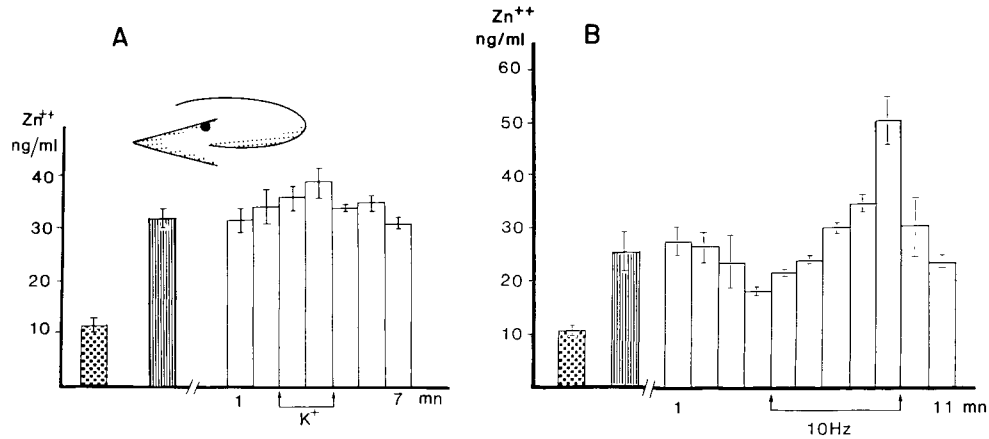

Fig. 3. Spontaneous and evoked release of zinc in the polymorph layer of the fascia dentata. A: spontaneous release, note that the concentration of zinc was 3 times higher than that found in the artificial CSF. K^+ failed to evoke a release of zinc. B: another experiment: a 10 Hz stimulus which induces a burst of population spikes (see Fig. 5), also evoked a release of zinc. Note the spontaneous release of zinc and the typical time course of the release observed in this region as compared to CA3 (e.g. Figs. 1A, B and 5).

other respects. Thus, we have observed in CA3 (c) only one case (out of 12) a small spontaneous release of zinc. In contrast spontaneous release of zinc was more frequent in the hilar zone (4 out of 7) (Fig. 3A).

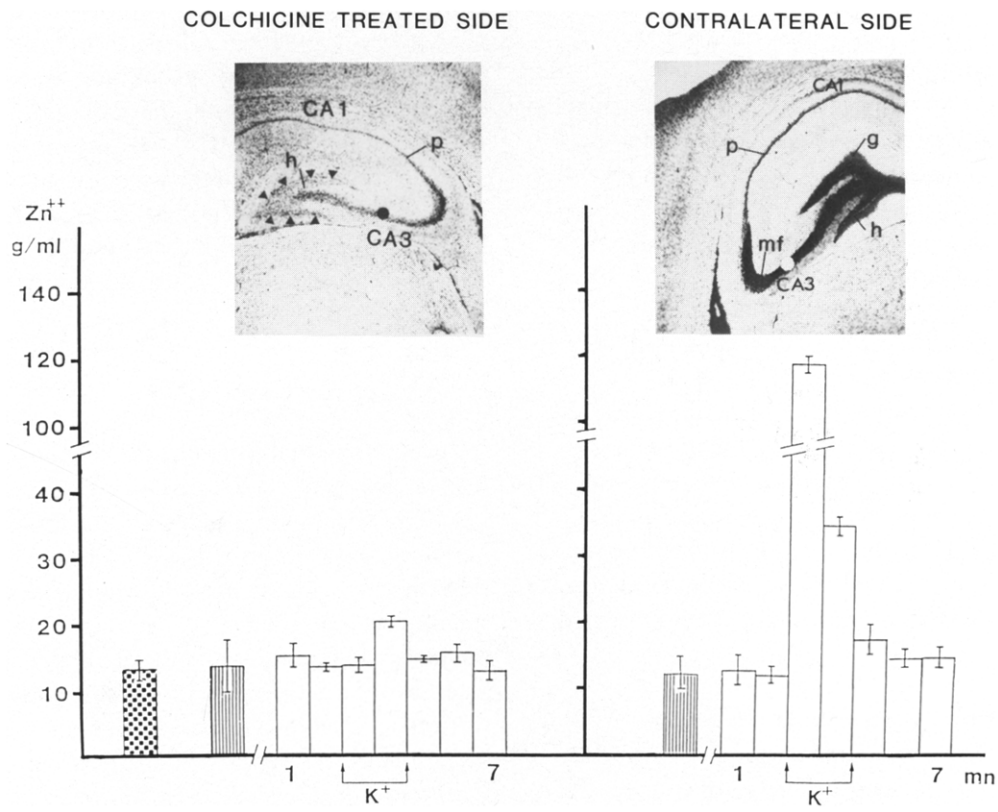

Fig. 4. Destruction of the granular layer and its mossy fibers prevents the K^+-evoked release of zinc. Colchicine, injected 6 days prior to the push–pull experiment, produced a complete destruction of the granular layer as shown in the Nissl and Timm staining photomicrograph. In the contralateral hippocampus K^+ induced a release of zinc. The location of the cannulae is indicated by circles.

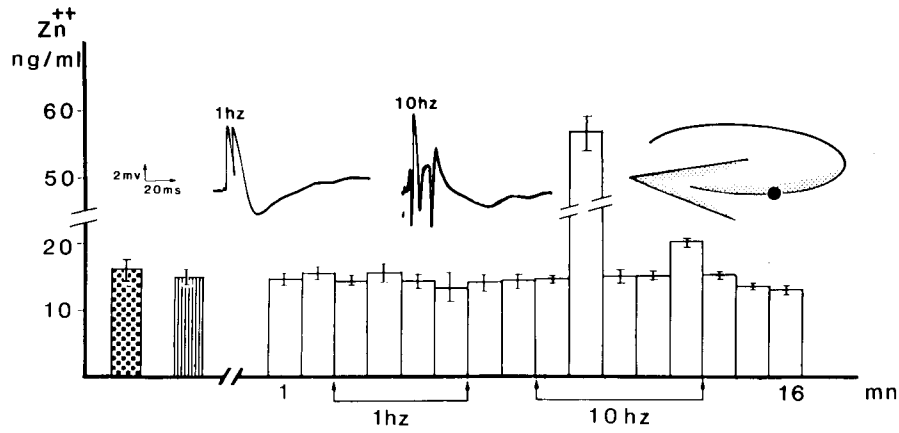

Fig. 5. Electrical stimulation of the perforant path produces a release of endogenous zinc in CA3. Stimulation at 1 Hz (1.5 times the threshold to elicit a population spike) evoked no release. In contrast a 10 Hz train which produced recurrent population spikes also evoked a release of zinc.

The magnitude of this release was 3–4-fold the levels of the CSF. Interestingly, spontaneous release was observed in 3 cases in which K^+ did not produce a release of zinc.

Effects of prior destruction of the mossy fibers

In agreement with other observations[17,26], injection of colchicine rapidly destroyed the granular neurons and their mossy fibers. A complete destruction can be observed within 4–5 days with an important glial proliferation in these layers (i.e. Fig. 4). With the procedure used in the present study, there was a complete destruction of the fascia dentata along the entire hippocampal axis. The Timm stain in the contralateral side was not reduced by this procedure and the pyramidal layer of the Ammon's horn was largely spared. In these conditions K^+ failed to produce a release of zinc in the colchicine treated hippocampi ($n = 6$). In 3 rats, in which the cannulae were adequately placed in the mossy fibers zone of both hemispheres, K^+ produced a release of zinc in the contralateral hemisphere but not in the colchicine treated side (Fig. 4).

Release of zinc by perforant path stimulation

Electrical stimulation of the perforant path at 1 Hz produced a typical negative population spike in the stratum lucidum of CA3 and in the hilus. This was not associated with a release of zinc. Similar observations were made in 5 rats. Increasing the frequency of stimulation to 10 Hz (5 min train) produced a burst of population spikes, this was consistently associated with a significant release of zinc 5–6-fold greater than the levels found in the control solution ($n = 5$) (Figs. 2B and 5). Interestingly, in CA3 this release pulse had a brief latency and duration, whereas in the hilus of the fascia dentata, the peak amplitude was progressively reached (Fig. 3B) and the response outlasted the duration of the K^+ pulse. In the other 4 cases, when the cannula was not placed in the hilus or in the stratum lucidum of CA3, the perforant path stimulation did not produce a release of zinc (Fig. 2B).

DISCUSSION

The present study provides compelling evidence that the mossy fibers selectively release endogenous zinc. This conclusion is based on the regional distribution of sites from which a K^+ pulse evokes a release of zinc, and on the observations that this response is also produced by electrical stimulation of the perforant path (and the mossy fibers) and eliminated by prior destruction of the mossy fibers. Both K^+-evoked release as well as that produced by electrical stimulation are exquisitely localized to the regions innervated by the mossy fibers (i.e. the stratum lucidum and the hilar zone); obviously there is very little diffusion of the zinc released from the mossy fibers. This explains the small rises of zinc produced by

K^+ in slices (two-fold the levels found in the control solution)[4]; since the mossy fibers represent approximately 8% of the volume of the hippocampus[15] and the diffusion of the agent is efficiently reduced by uptake[21], a release restricted to the mossy fibers is not expected to produce a large increase in the zinc concentrations in the slice perfusate. The present study also suggests important differences between stratum lucidum of CA3 (a, b, c) and the polymorph zone of the fascia dentata. In CA3 there is no spontaneous release and the evoked release of zinc has a short latency and a brief duration. Also, this release can reach particularly high concentrations (up to 2000 ng/ml), which is as much as 15% of the estimated content of zinc in the mossy fiber terminals (220–300 μM)[15]. In the polymorph zone, where collaterals of the mossy fibers innervate pyramidal basket cells[16], zinc is spontaneously released and the evoked release is relatively small (maximum 5-fold increase) and has a longer duration and latency to peak. In keeping with this, there are some indications in the literature that the concentration of zinc is higher in the mossy fibers of CA3 than in the polymorph layer[15]. It is possible that these differences reflect a stronger uptake in CA3 than in the polymorph zone. Further information on the fine morphology of the mossy fibers in both areas is required to explain the significance of these differences.

In conclusion zinc in the mossy fibers fulfills most of the criteria required to suggest an involvement in synaptic transmission, including a selective localization in terminals and an uptake and release upon stimulation. Neither the mechanism of action, nor the role of zinc in this system have been clarified. Furthermore the transmitter released from the mossy fibers has not been fully characterized, and the nature of the protein which could be released with zinc is currently unknown. Interestingly, several observations suggest that the mossy fibers release a nerve growth factor (NGF)-like protein[8] (but also see Heacock et al.[19]) and zinc is a cofactor of the NGF[28]. It is also interesting that the pyramidal cells of CA3 are the most susceptible region to epileptogenic agents and procedures[5], and i.c.v. injections of zinc produce a hippocampal seizure[27]. Clearly a better understanding of the role of zinc in the synaptic transmission of the mossy fibers is of particular interest. The methodology used in the present studies (i.e. a combination of push–pull and electrophysiological methods in the intact animal), is highly suitable to characterize the release of agents in localized zones of the hippocampus.

ACKNOWLEDGEMENTS

Financial support from DRET (no. 84/1429) is acknowledged.

REFERENCES

1 Albe-Fessard, D., Stutinsky, F. and Libouban, S., *Atlas Stéréotaxique du Diencéphale du Rat Blanc,* CNRS publication, Paris, 1971.
2 Andersen, P., Holmquist, B. and Voorhoeve, P.E., Entorhinal activation of dentate granule cells, *Acta Physiol. Scand.,* 66 (1966) 448–460.
3 Aniksztejn, L., Charton, G. and Ben-Ari, Y., In situ release of endogenous zinc from the mossy fibers in the hippocampus, *Epilepsia,* 26 (1985) 513.
4 Assaf, S.Y. and Chung, S.H., Release of endogenous zinc from brain tissue during activity, *Nature (London),* 308 (1984) 734–736.
5 Ben-Ari, Y., Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy, *Neuroscience,* 14 (1985) 375–403.
6 Charton, G., Rovira, C., Ben-Ari, Y. and Leviel, V., Spontaneous and evoked release of endogenous zinc in the hippocampal mossy fibers zone of the rat in situ, *Exp. Brain Res.,* 58 (1985) 202–205.
7 Chung, S.H. and Assaf, S.Y., Emerging roles of zinc ions in neuronal transmission. In C.G. Frederickson, G.A. Howell and E.J. Kasarkis (Eds.), *The Neurobiology of Zinc, Part. A,* Alan R. Liss, New York, 1984, pp. 174–189.
8 Collins, F. and Crutcher, K.A., Neurotrophic activity in the adult rat hippocampal formation: regional distribution and increase after septal lesion, *J. Neurosci.,* 5 (1985) 2809–2814.
9 Crawford, I.L. and Connor, J.D., Zinc in maturing rat brain: hippocampal concentration and localization, *J. Neurochem.,* 19 (1972) 1451–1458.
10 Dreosti, I.E., Zinc in the central nervous system: the emerging interactions. In C.G. Frederickson, G.A. Howell and E.J. Kasarkis (Eds.), *The Neurobiology of Zinc, Part. A,* Alan R. Liss, New York, 1984, pp. 1–26.
11 Ebadi, M., White, R.W. and Swanson, S., The presence and function of zinc binding proteins in developing and mature brains. In C.G. Frederickson, G.A. Howell and E.J. Kasarkis (Eds.), *The Neurobiology of Zinc, Part. A,* Alan R. Liss, New York, 1984, pp. 28–39.
12 Fjerdingstad, E., Danscher, G. and Fjerdingstad, E.J., Changes in zinc and lead content of rat hippocampus and whole brain following intravital dithizone treatment as de-

termined by flameless absorption spectrophotometry, *Brain Research,* 130 (1977) 369–373.
13 Frederickson, C.J., Howell, G.A. and Frederickson, M.H., Zinc dithizonates relationship to the mossy fiber neuropil and postnatal development, *Exp. Neurol.* 73 (1981) 812–823.
14 Frederickson, C.J., Howell, G.A. and Kasarkis, E.J., *The Neurobiology of Zinc, Part. A,* Alan R. Liss, New York, 1984, 390 pp.
15 Frederickson, C.J., Klitenick, M.C., Manton, W.I. and Kirkpatrick, J.B., Cytoarchitectonic distribution of zinc in the hippocampus of man and the rat, *Brain Research,* 273 (1983) 335–339.
16 Frotscher, M., Mossy fibres form synapses with identified pyramidal basket cells in the CA3 region of the guinea-pig hippocampus: a combined Golgi-electron microscope study, *J. Neurocytol.,* 14 (1985) 245–259.
17 Goldschmidt, R. and Steward, O., Preferential neurotoxicity of colchicine for granule cells of the dentate gyrus of the adult rat, *Proc. Natl. Acad. Sci., U.S.A.,* 77 (1980) 3047.
18 Haug, F.M.S., Electron microscopical localization of the zinc in hippocampal mossy fiber synapses by a modified sulphide silver procedure, *Histochimie,* 8 (1967) 355–368.
18a Havy, F.M.S., Heavy metals in the brain. A light microscope study of the rat with Timm's sulphide silver method. Methodological considerations and cytological and regional staining patterns, *Adv. Anat. Embryol. Cell. Biol.,* 47 (1973) 1–71.
19 Heacock, A.M., Schonfeld, A.R. and Katzman, B., Hippocampal neurotrophic factor: characterization and response to denervation, *Brain Research,* 363 (1986) 299–306.
20 Hesse, G., Chronic zinc deficiency alters neuronal function of hippocampal mossy fibers, *Science,* 205 (1979) 1005–1007.
21 Howell, G.A., Welch, M.G. and Frederickson, C.J., Stimulation induced uptake and release of zinc in hippocampal slices, *Nature (London),* 308 (1984) 736–738.
22 Hu, K.H. and Friede, R.L., Topographic determination of zinc in human brain by atomic absorption spectrophotometry, *J. Neurol.,* 15 (1968) 677–685.
23 Ibata, Y. and Otsuka, N., Electron microscopic demonstration of zinc in the hippocampal formation using sulphide silver technique, *J. Histochem. Cytochem.,* 17 (1969) 171–175.
24 Khulusi, S.S., Brown, M.W. and Wright, D.M., Zinc and paired-pulse potentiation in the hippocampus, *Brain Research,* 363 (1986) 152–155.
25 Maske, H., Über den topochemischen Nachweis von Zink in Ammonshorn verschniedener Saugetiere, *Naturwissenschaften,* 42 (1955) 424.
26 Nadler, J.V. and Cuthbertson, G.J., Kainic acid neurotoxicity toward hippocampal formation: dependence on specific excitatory pathways, *Brain Research,* 195 (1980) 47–56.
27 Pei, Y., Zhao, D., Huang, J. and Cao, L., Zinc-induced seizures: a new experimental model of epilepsy, *Epilepsia,* 24 (1983) 169–176.
28 Pattison, S.E. and Dunn, F., On the relationship of zinc ion to the structure and function of the 7S nerve growth factor protein, *Biochemistry,* (Vol. 14), 12 (1975) 2733–2738.
29 Sloviter, R.S., A simplified Timm stain procedure compatible and routine paraffin embedding of rat brain, *Brain Res. Bull.,* 8 (1982) 771–774.
30 Von Euler, C., On the significance of the high zinc content in the hippocampal formation, *Physiologie de l'Hippocampe Colloques Internationaux du CNRS,* Ed. CNRS, Paris, 1962.