Zinc and Hippocampal Function

I. L. Crawford and J. D. Connor[1]

INTRODUCTION

Zinc and Hippocampal Cytoarchitecture

Considerable attention has been focused recently on the hippocampus as a brain region in which a relationship between a trace metal and neuronal function may be amenable to investigation. Maske (1955) made the initial observation that a metal was apparently localized in high concentration within this phylogenetically old and relatively large cortical area. While using an intravital chelating agent, dithizone (diphenylthiocarbazone), in a study of pancreatic zinc, Maske fortuitously examined the brains of his experimental animals. He noted that parts of the hippocampal formation had a highly selective affinity for the stain and suggested that the chromophore resulted from deposition **in vivo** of a zinc dithi-zonate complex. This idea was supported by subsequent observations that ^{65}Zn accumulates in the same hippocampal area as that stained by dithizone (von Euler, 1961; Hassler and Soremark, 1968).

FIGURE 1

Hippocampal region of the cat, Timm's silver sulfide stain for metals, thionin counterstain. Neurons and pathways of reference in ink. Mossy fibers (mf) arise from granule cells (GC) in the fascia dentata (granule somata and dendrites) and project to the apical dendrites of pyramidal cells (e.g., CA3, but not CA1). The perforant pathway (pp) consists of the axons of neurons whose cell bodies lie in the entorhinal cortex (Entor. Cx). These axons synapse with the dendrites in the molecular layer of the fascia dentata. Afferent fibers of the commissural pathway (cp) originate from pyramidal cells in the contralateral hippocampus and terminate on basal dendrites of pyramidal cells (CA3). Calibration (photomicrograph only):500u.

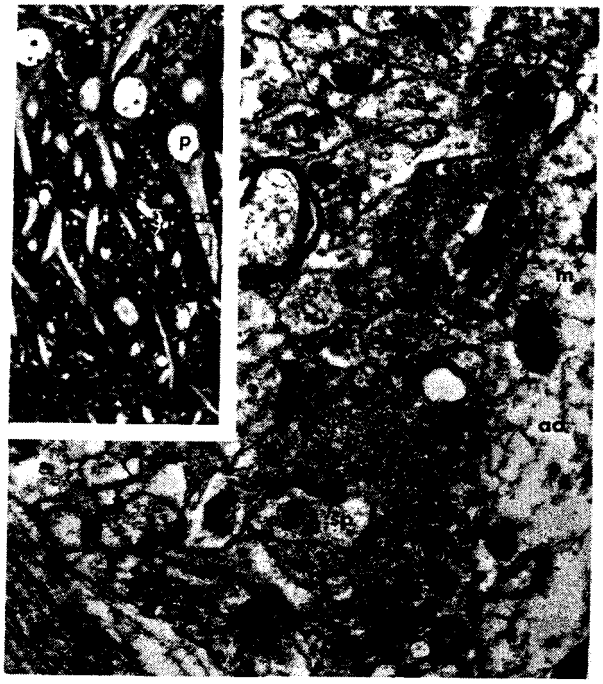
[1] Department of Pharmacology, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033. *(Supported by USPHS Grant NS 08884, NINDS)

Tissues stained intravitally by dithi-zone are unsuitable for high power light microscopy, hence the stained cellular elements could not be precisely identified. For this reason, zinc was first thought to be localized within the perikarya of pyramidal and granule cells, since dithizone seemed to bind within these two prominent layers (Fleischhauer and Horstman, 1957). This question was reopened by Timm (1958). He developed a histochemical method with resolution better than that of dithizone and found that heavy metal staining was actually most intense in a zone subjacent to the cell body laminae. McLardy (1960) and von Euler (1961) pointed out the remarkable correspondence between the location of the stained area and the mossy fiber system described by early neuroanatomists.

Mossy fibers form an important intrahippocampal pathway (Figure 1) connecting the fascia dentata with the regio" inferior (Ramon y Cajal, 1893). The pathway consists of axons of granule cells whose terminal fields are restricted to the CA3-4 pyramidal cells in the regio inferior (Lorente de No', 1934). Ultra-structural studies have indicated that mossy fibers synapse **en passant** with apical dendrites of CA3-4 pyramids; their large boutons have a distinctive morphology (Blackstad and Kjaerheim, 1961; Hamlyn, 1961) which is evident in Figure 2.

By combining electron microscopy with a modification of Timm's silver sulfide method, Haug (1967) found that the cytoplasm and vesicles of mossy fiber boutons were associated with punctate metallic deposits. The electron dense particles were absent from terminal mitochondria, dendritic spines projecting into the boutons, and. the surrounding axonal, glial, and vascular elements. The mossy fiber pathway is the only neuronal system in the brain for which enough histochemical evidence is currently available to suggest that a close relationship may exist between a specific trace metal and synaptic function (Ibata . and Otsuka, 1969).

FIGURE 2



Ultrastructure of mossy fiber boutons. A. Low power photomicrograph (1200 X) of a pyramidal cell (p) and the trunk of its apical dendrite (ad). Epon section stained with Azure II and Toluidine Blue. The designated area along the dendrite represents the approximate location of the material in the electronmicrograph. B. Fine structure (30,000X) of a bouton (mfb) in apposition to the apical dendrite (ad). Mossy fiber boutons are exceptionally large, are derived from unmyelinated axons (a), and are filled with many small (~400Å), clear vesicles (v). Mitochondria (m) are present in the presynaptic and postsynaptic elements. Synaptic contacts (arrows) are prominent on dendritic spines (sp) projecting into the boutons. Thin epon section, after glutaraldehyde-osmium fixation (in collaboration with A. Leure-duPree and J.M. Rosenstein, Department of Anatomy, M.S. Hershey Medical Center).

Regional Zinc Concentrations

Quantitative chemical analyses have consistently supported the histochemical evidence that zinc is differentially distributed in brain. One would expect **a priori** that the zinc content of the hippocampal region might be large, since the mossy fiber system occupies a considerable portion of the hippocampal mass and is zinc-rich. This is, in fact, the case. In the rabbit (Klee and Lieflander, 1965), cow, pig (Wong and Fritze, 1969), rat (Crawford and Connor, 1971), and human (Hu and Friede, 1968), the concentration of zinc in the hippocampus has been shown by quantitative measurement to be larger than other brain areas. An uneven distribution of an

endogenous substance is often taken as an indication that the substance subserves specialized functions in the regions of high concentration. By way of contrast, other transitional metals such as copper and manganese are more concentrated elsewhere in the nervous system (Hanig and Aprison, 1967; Donaldson et al., 1973).

Unfortunately, whole hippocampal analyses cannot distinguish between the zinc in the mossy fiber boutons and that in other cellular elements. This problem has been partially resolved by determining the subcellular distribution of zinc in homogenates of rat hippocampus after differential centrifugation (Crawford and Connor, 1972). More than half of the total hippocampal zinc was located in a pellet where large boutons would be expected. Another way of estimating the ratio of mossy fiber to total hippocampal zinc is to measure the metal concentration in developing animals. The working hypothesis in this instance is that the zinc concentrations in the mossy fibers at some early maturational stage is approximately the same as that of surrounding tissues, but measurable changes in the metal occur during growth.

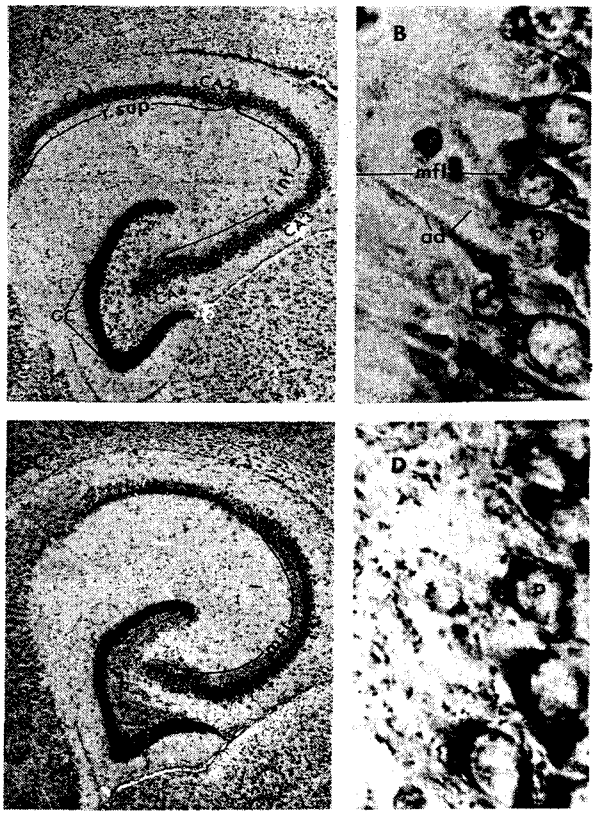
ZINC IN MATURING HIPPOCAMPUS

Metallohistochemistry

During histogenesis the structural and functional components of neurons arise in ordered sequence, each succeeding event being dependent upon successful completion of a preceding stage. Changes in morphology may thus signal congruent onset of some particular function. Generalizations such as these have been useful in discerning a possible role for zinc in the postnatal maturation of granule cells.

Fleischhauer (1957), while characterizing the effects of acute administration of dithizone, noted that the hippocampus of rat pups did not bind the stain until about 20 days after birth. This provocative observation was subsequently explored in detail with Timm's silver sulfide method (Crawford and Connor, 1972). In very young rats, hippocampal staining was light, diffuse, and generally unremarkable until 18 days after birth, at which time the fascia dentata and its hilus were darker than other brain areas. An intense burnt orange color in the hilus and mossy fiber layer, characteristic of the adult hippocampus, was fully developed at 22 days postpartum (Figure 3). Long-term studies have indicated that when the adult staining pattern is established, there is apparently little subsequent change in stain reactivity (Brun and Brunk, 1973). These observations suggest that mossy fiber zinc undergoes abrupt changes in concentration, localization, or availability for reaction during granule cell maturation.

FIGURE 3



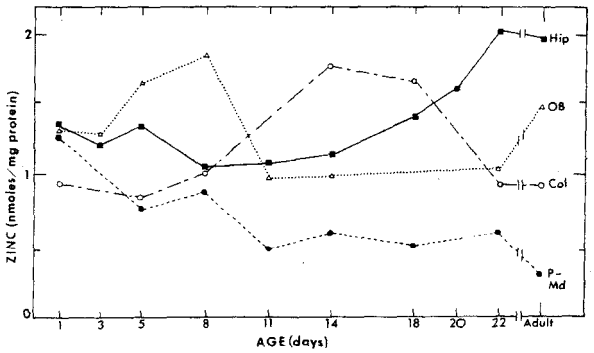
Comparative metallohistochemistry of hippocampal regions in 18 and 22 day old rats. Horizontal sections, silver sulfide stain (Timm, 1958), thionin counterstain; tissues processed simultaneously. A. At 18 days (60X): granule cells (GC), pyramidal cells (CA3-4) or regio inferior (r.inf.), neuronal elements in the concavity of the regio inferior were not stained at this age. B. Higher magnification (1500X) of regio inferior, same section as A: pyramidal soma (p), apical dendrite (ad), area of the mossy fiber layer (mfl). C. At 22 days (60X); note stain deposited in hilus (h) of fascia dentata and in the mossy fiber layer. D. Pyramidal cells and mossy fiber layer (1500X), same section as C; note profuse deposits of reaction product subjacent to the perikarya.

Postnatal Zinc Concentrations

In parallel with the histochemical studies, age-dependent alterations in zinc levels in maturing rat hippocampus and other brain regions were determined by atomic absorption spectrophotometry (Figure 4). The hippocampus was the only area in which the zinc content increased significantly ($p<0.005$) between 14 and 22 days to reach adult levels. The pattern for whole brain zinc as a function of age was different (Table 1). Whole brain zinc remained almost constant after 11 days, a result similar to the observations of Koford (1970). Hip-pocampal zinc was significantly different from the whole brain after the 14th postnatal day, but not before.

Chemical analyses of large brain samples are subject to the criticism that pooled data are not referable to specific cellular structures. Furthermore, zinc is undoubtedly essential for the growth and maintenance of many cellular components (Hurley and Shrader, 1972; Sandstead et al., 1972). For these reasons it is difficult to speculate at length on how much of the large increment in total hippocampal zinc, which accrues post-natally during the 14-22 day epoch, actually represents incorporation into mossy fibers. Among the important developmental questions about the hippocampus which remain unanswered are the mechanism for the rapid accumulation of zinc in the third week of life and the cytological location of the metal during the earlier periods of ontogeny.

FIGURE 4



Concentrations of zinc in hippocampus and three other regions of rat brain as a function of age. Individual values are the means of two or more assays not differing from the mean by more than 15 percent (tissues from 10 to 30 rats for each analysis). The high levels in the olfactory bulb and colliculi of early age groups were not accompanied by notable metallohistochemical staining. Abbreviations: Hip, Hippo-campus; OB, olfactory bulb; Col, colliculi; P-MD, pons-medulla. (For data on other brain regions see Crawford and Connor, 1972).

TABLE 1

Comparison of Zinc Concentrations in Hippocampus and Whole Brain of Maturing Rat [a]

AGE (days)	ZINC (pmoles/mg protein) Hippocampus[b]	Whole Brain	2P[c]
1-3	1333 ± 48	1165 ± 52	> 0.050
5-8	1230 ± 87	1085 ± 44	> 0.050
11-14	1114 ± 100	850 ± 76*	< 0.050
18-20	1383 ± 125(4)	890 ± 55(4)	< 0.010
22	2108 ± 84**	860 ± 49(7)	< 0.005
> 90 (Adult)	1938 ± 50(4)	780 ± 25(7)	< 0.005

[a] Values were obtained from wet-ashed tissue homogenates by atomic absorption spectrophotometry. Means ± S.E. are given for three assays unless otherwise indicated in parentheses. Protein estimated by a biuret method.

[b] Tissues from 10-30 rats were required for each analysis.

[c] Compared by two-sample t test.

* 2P < 0.05, compared to values at previous age; ** 2P < 0.005.

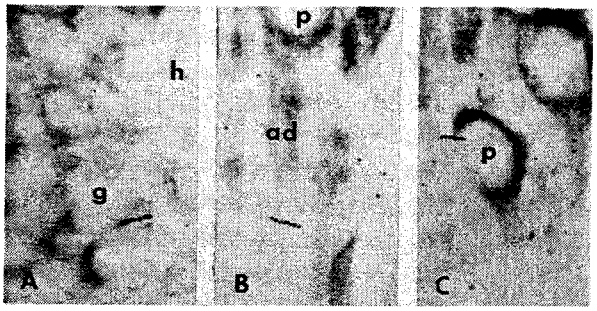
^{65}Zn and Granule Cell Morphogenesis

Specific structural elements in the immature hippocampus which take up zinc during development have been identified by autoradiography (Crawford and Connor, 1972). Rat pups of various ages were injected systemically with ^{65}ZnCI 18 hours before sacrifice. The locations of positron tracks were studied in thin brain sections after several weeks exposure (Figure 5). The number of emission tracks in the mossy fiber, pyramidal, and granule cell layers were recorded at different stages of maturation (Figure 6). Certain trends were apparent in the postnatal uptake and distribution of exogenous zinc; however, these data were not subjected to statistical tests because the number of tracks at certain ages was small.

In the youngest rats, most of the tracks were in the granule cell layer; after 18 days of age a proportionately greater number of tracks emanated from the mossy fiber layer. These chronological changes in the location of zinc, as well as the differences in histochemical reactivity, may be related to the morphogenesis of the granule cell. The differentiation and development of these interneurons are thought to occur after birth in the rat (Altman and Das, 1965). The important postnatal phases are: 1. proliferative mitosis of the cells of origin in the ependymal lining of the ventricle, 2. orderly migration past the apical dendritic field of CA3-4 pyramids, with the formation of synaptic junctions there, 3. continued migration of the perikarya into the fascia dentata by axonal elongation, 4. congregation of granule cell somata into a specific lamina. This sequence is completed by the end of the third postnatal week (Altman, 1966), a time which coincides with marked changes in the concentration of zinc in the granule cell axons, a shift in the location of exogenously administered isotopic zinc, and the onset of histochemical reactivity.

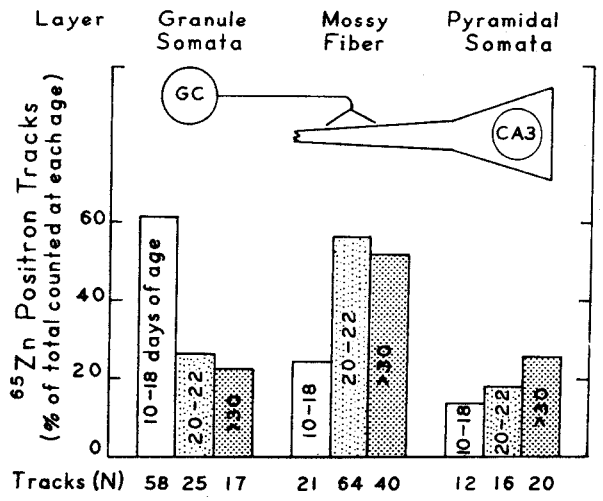
Taken together, these observations indicate that the accumulation of zinc by mossy fibers is part of a general series of events in the maturation of granule cells. Whether or not the completion of these morphological and neurochemical events are causally related to the onset of impulse conduction and neurotransmission in the mossy fiber pathway remains to be determined. Nevertheless, the precisely timed appearance of zinc within mossy fiber boutons probably represents a critical stage in hippocampal maturation and thus requires careful consideration in any correlation of structure with function.

FIGURE 5



Localization of ^{65}Zn in the hippocampus of maturing rats after systemic administration (1 υ Ci/g). Positron tracks in autoradiographic preparations lightly stained with thionin. A. Track in granule cell layer (g) near hilus (h) of the fascia dentata at 18 days, the most frequent site of isotopic emissions at or before this age. B. Track overlying an apical dendrite (ad) in the mossy fiber layer subjacent to pyramidal somata (p), 22 days postpartum. After 20 days, the majority of tracks were in this region. C. Track in proximity to a pyramidal cell body, 30 days of age.

FIGURE 6



Distribution of isotopic zinc in the mossy fiber system of the rat at three stages of development. The number of tracks in each of the three layers were summed for each postnatal period. The heights of the bars represent the percentage contributed by each layer to the total number of counts at each stage.

HIPPOCAMPAL AND GRANULE CELL FUNCTIONS

Physiologic Role of the Hippocampus

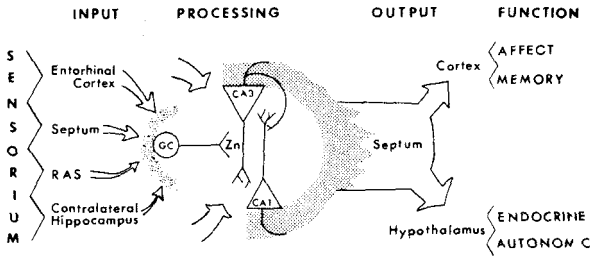
Morphological and neurochemical evidence raises the prospect that the contribution of the hippocampus to complex brain processes may be partially dependent upon the availability of zinc. This possibility has wide implications because of the key position of the hippocampus within the limbic system (Broca, 1878; Green, 1964). By means of polyneuronal connections with the neocortex and hypothalamic nuclei (Papez, 1937), the hippocampus has the potential for strongly influencing behavioral and viscerosomatic activities (MacLean, 1970).

While the precise role of the hippocampus within the realm of cerebral function is poorly understood, the results of experimental and clinical studies leave little doubt that stimulation or ablation of the hippocampus alters higher nervous system responsiveness. The reciprocal relationship between hippocampal theta rhythms and some states of arousal (Green and Arduini, 1954) has given rise to the idea that the hippocampus influences affective behavior by diffuse regulation of cerebral excitability (Parmeggiani, 1967). Retrograde loss of memory for recent events and impaired retention of information after bilateral hippocampectomy (Scoville and Milner, 1957; Isaacson, 1972) suggest that the hippocampus participates in memory processing. Another proposed function is the modulation of neuroendocrine secretions via the hypothalamus (Mason et al., 1961), since hippocampal stimuli suppress the pituitary-adrenocortical axis (Rubin et al., 1966). In addition, the recent work of Kawakami and Kubo (1971) on the electrophysiological responsiveness of the hippocampus to small fluctuations in circulating hormones points to the possibility of cybernetic regulatory mechanisms. The hippocampus is also presumed to participate in autonomic modulation, since stimuli in discrete hippocampal areas evoke reproducible responses in the cardiovascular, gastrointestinal, and genitourinary systems (MacLean, 1960; Hockman et al., 1969). The burden of this discussion is that alterations in the concentration or location of hippocampal zinc could be manifested in a variety of ways, all related to the plethora of brain activities under hippocampal influence (Figure 7).

Converging Afferent Pathways

Neuronal information derived from transducers in contact with the internal and external environment is eventually conveyed via fimbrial or perforant pathways into the hippocampus. Primary sensory afferents do not impinge directly on hippocampal neurons; however the structure is closely connected with important sensory relay and integrative areas. Electrophysiological responses (e.g., changes in spontaneous slow-wave activity, evoked potentials, neuronal discharge patterns, and transmembrane potentials) have been recorded in the hippocampus during stimulation of the entorhinal cortex (Renshaw et al., 1940; Lomo, 1971), septum (Stumpf, 1965), reticular activating system (Grantyn and Grantyn, 1972), and contralateral hippocampus (Andersen, 1960). These

FIGURE 7



Diagrammatic survey of hippocampal function with emphasis on the mossy fiber system. The hippocampus receives sensory information (olfactory, visual, auditory, visceral, tactile, etc.) through the perforant (from entorhinal cortex only) and fimbrial (from numerous areas) pathways. Afferent impulses influence pyramidal cells (e.g. CA3, CA1) either directly or via granule cells (GC) and their zinc-rich mossy boutons. The mossy fibers, by modulating the activity of some pyramidal cells, participate in the integration of information. Pyramidal axons conduct impulses to or through the septum and represent the only projection from the hippocampus to other brain regions.

studies indicate that the hippocampus monitors and responds to the activity of other brain areas by integrating converging impulses.

Some afferent axons enter the hippocampus and synapse directly on pyramidal cells. Others terminate on the dendrites of granule cells in the molecular layer of the fascia dentata (Blackstad, 1967). An example of a large fascicle in the latter category is the perforant pathway which originates from neurons in the entorhinal cortex. Electrical stimuli in the entorhinal area evoke short latency excitatory postsynaptic potentials in granule cells (Andersen and Lomo, 1967). Information entering the hippocampus through the dentate is eventually represented by activity in the mossy fiber relay.

Mossy Fiber Activation

Cajal, in his description of hippocam-pal cytology (1893), surmised that the mossy fibers, because they form the only association pathway between the fascia dentata and pyramidal cells of the regio inferior, would be of considerable functional importance. This conclusion was corroborated by subsequent physiological investigations. Axons of diverse origin converge on granule cells which in turn excite only a restricted population of pyramidal cells (Andersen et al., 1966). The output of the granule interneuron is circumscribed, highly localized, and excitatory (Figure 7).

Neurotransmission mediated through the zinc-rich mossy fiber boutons can be studied by physiologically activating the mossy fiber pathway (stimulation of entorhinal cortex) while recording the responses of pyramidal cells in the CA3-4 region. In this regard, the evoked potential is a useful technique because it represents the summed responses of many pyramidal neurons and can be easily utilized in unanesthetized animals. Since mossy fiber synapses are limited to apical dendrites in a distinct layer, their activation creates fields of current flow in the pyramidal cell layer. In response to mossy fiber depolarization, measurable voltage fluctuations, or evoked potentials, can be detected on the surface of the hippocampus. In contrast to the mossy fibers, the terminals of the excitatory commissural pathway, which are not zinc-rich, synapse on the basal dendrites of CA3-4 pyramids (Blackstad, 1967). Thus, access is available for depolarizing the same cells by two different pathways, only one of which contains notable concentrations of zinc. This difference can be exploited to elucidate the functional participation of zinc in excitatory neurotransmission.

NEUROPHARMACOLOGY OF HIPPOCAMPAL ZINC

Effects of Dithizone on Evoked Waves

The selective intravital affinity of dithizone for mossy fibers provides an opportunity for determining the effects of zinc chelation on mossy fiber function. The effects of systemic injections of the chelator on hippocam-pal responses to mossy fiber activation were studied in unanesthetized rats (Crawford et al., 1973). The effects of the compound on the responses to commissural pathway stimulation were also studied as a test for specificity.

Acute and subacute effects of dithizone were examined in awake, unrestrained rats with recording electrodes chronically implanted in their hippocampi. Stimulating electrodes in the entorhinal cortex and at a homotopical point in the contralateral hippocampus were used to evoke waves through the mossy fiber and commissural pathways, respectively (Figure 8).

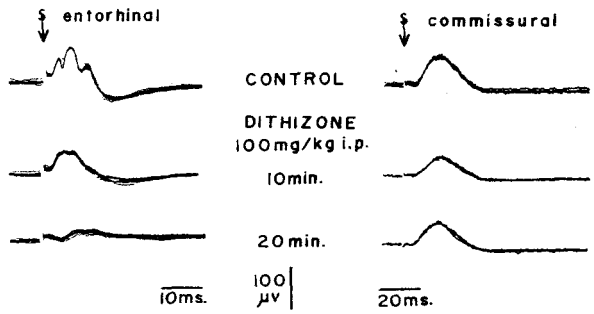
According to Andersen et al.(1971), the negative potentials represent depolarization of a pyramidal population. Low doses of dithizone (25 mg/kg) potentiated slightly the late component of the complex waveform evoked by entorhinal timuli; somewhat larger doses (50 ng/kg) reduced the amplitude of all parts of the wave. These drug effects diminished gradually, until they were imperceptible 45 minutes after adminis-tration.

Doses of 100 mg/kg depressed the wave within 10 minutes after injection. Onset of sustained wave block occurred by 20 minutes, and the blockade persisted for three hours or more. The drug had no effect on waves evoked by commissural stimulation, except for a short duration depression at the 100 mg/kg dose (Figure 9).

Multiple Injections of Dithizone

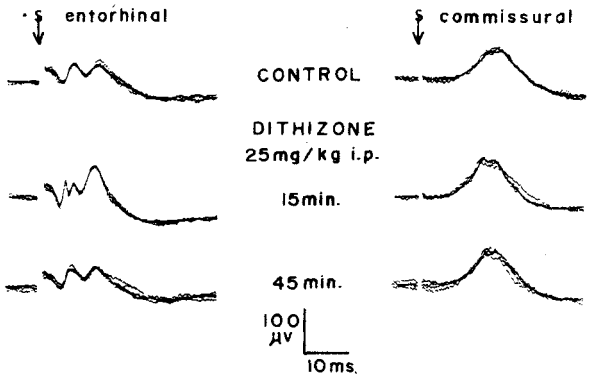
Danscher and Haug (1971) reported that intermittent treatment with dithi-

FIGURE 8



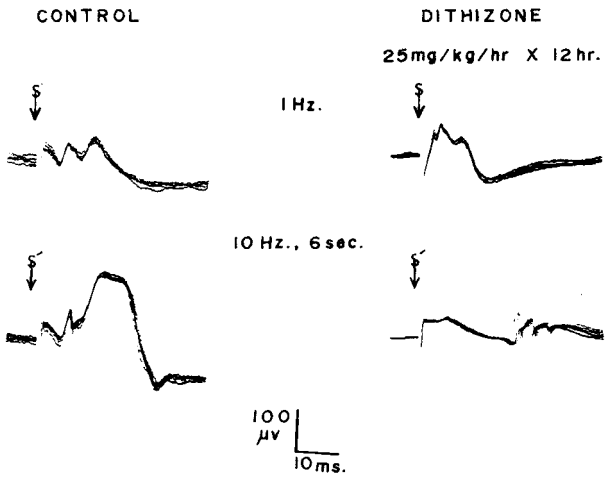
Acute effects of a small dose of dithizone on evoked waves in the rat hippocampus. Oscilloscope records (multiple superimposed sweeps, negativity up) from an electrode in the hippocampus of an unanesthetized, adult, male rat; three days after bipolar electrode implantation. Stimuli (arrows; 25 volts, 0.5 ms duration) were delivered alternately (1Hz) to the entorhinal cortex (mossy fiber activation) and to the contralateral hippocampus (commissural activation). Note the potentiation in the late negative component of the wave evoked by entorhinal stimuli 15 min after dithizone. Placement of electrodes was verified histologically.

FIGURE 9



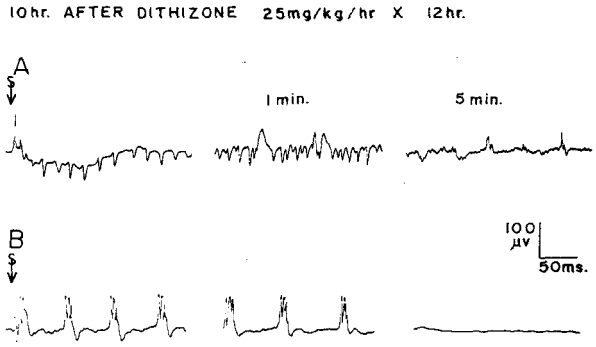
Acute effects of a large dose of dithizone on hippocampal waves evoked by afferent stimuli. Experiment similar to that of Figure 8. Stimuli in this case were 15 and 20 volts, entorhinal and commissural respectively. Contrast the marked depression of the potential evoked by entorhinal stimulation with minimal effects on commissural response.

FIGURE 10



Effects of multiple doses of dithizone on the response to a single stimulus before and after repetitive entorhinal stimuli. Single stimuli (1 Hz; 20 volts, control; 40 volts, dithizone) continued to evoke mossy fiber mediated responses after dithizone treatment. However, the potentiated response to single stimuli which follows high frequency stimulation (10 Hz, for 6 seconds, 40 volts) was absent after the dose regimen.

FIGURE 11



Epileptiform activities recorded from the hippocampus of two rats (A and B) in response to single entorhinal stimuli after repeated injections of dithizone. A. Repetitive spiking of several minutes duration after a single stimulus. B. Spikes with superimposed afterdischarges. Note regularity of isoelectric postictal periods (single oscilloscope traces; stimulus intensity; A. 35 volts, B. 20 volts).

zone depleted the mossy fibers of histochemically reactive metals. This suggests that frequent injections of the drug might change the physiological responses mediated by the pathway. The hypothesis was tested by injecting 25 mg/kg/hr intraperitoneally for 12 hours into unanesthetized rats with chronically implanted electrodes (Crawford et al., 1973). During and immediately after the dose regimen, single entorhinal stimuli

evoked rather typical mossy fiber responses, although somewhat stronger stimulus intensities were required (Figure 10). Two changes in electrophysiological responsiveness were observed as a result of repeated injections of dithizone. Firstly, the potentiated response to single stimuli after a stimulus train (Andersen and LoVno, 1967) was absent following the sixth dose (Figure 10). Secondly, 10 hours after the last dose, single stimuli produced multiple electrographic spikes, usually accompanied by after-discharges (Figure 11).

In agreement with the results of Danscher and Haug, the intravital staining which normally follows 100 mg/kg of dithizone was completely prevented by repeated smaller doses of the drug. Moreover, by quantitative analyses, the zinc concentration in the whole hippocampus was reduced by 20 percent after multiple dithizone injections.

Effects of Other Agents

Evidence for a selective block of hippocampal transmission **in vivo** was obtained by von Euler (1961). He found that a saturated solution of hydrogen sulfide in isotonic saline applied to the hippocampal surface irreversibly blocked the CA3 pyramidal cell responses mediated through mossy fibers.

Neurotransmission between mossy fibers and CA3 neurons has also been studied in thin slices of brain (Yamamoto, 1972). The late wave of the field potential evoked in vitro was correlated temporally with excitatory potentials recorded intracellularly from pyramidal cells. The conclusion that the late component represented chemical transmission was supported by the observation that the late negative wave was depressed by increasing the Mg+ + or reducing the CA + + concentrations in the medium. In both in vitro and in vivo studies, a variety of pharmacologic agents including atropine, nicotine, and phentolamine exert variable and nonspecific actions on potentials elicited by excitatory afferents to the hippocampus (Izquierdo and Izquierdo, 1971).

A number of studies have dealt with a different measure of responsiveness, namely the effects of systemic administrations of drugs on the metallohisto-chemistry of mossy fibers. Single intraperitoneal injections of 100 mg/kg of either alloxan or oxine (8-hydroxyqui-noline) intensified the Timm's stain in the hippocampus of rats (Otsuka and Ibata, 1968). Larger doses of oxine, 400 mg/kg, reduced the intensity of the silver sulfide reaction (Danscher and Fredens, 1972). Disulfiram or its metabolite, diethyldi-thiocarbamate, also diminished the Timm's reaction. All of these compounds are metal chelators and presumably alter the histochemistry of the mossy fibers by removing or sequestering zinc. It is conceivable that, like dithizone, many chelators alter synaptic transmission in the hippocampus, although this remains to be determined. Drugs may also interact with mossy fibers in other ways; for example, phenothiazines reportedly increased the uptake **in vivo** of ^{65}Zn into the hippocampus (Czerniak and Haim, 1971).

ZINC AND OTHER ENDOGENOUS SUBSTANCES

Hypothetical Roles for Zinc in Mossy Fibers

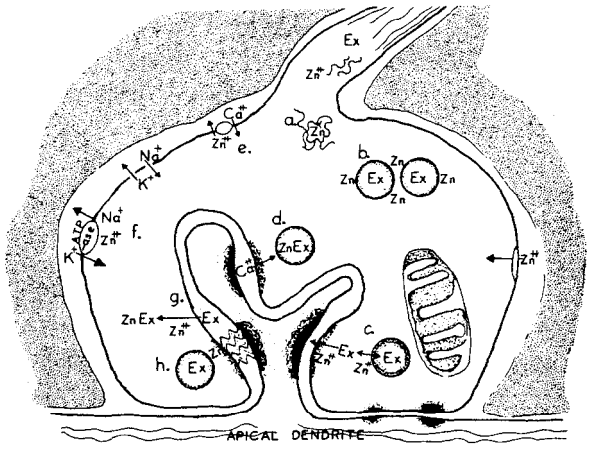
The synaptic mechanisms with which zinc is associated within mossy fiber boutons, and the endogenous substances therein with which zinc interacts, are as yet unidentified. Several possible molecular relationships which might merit further investigation are outlined in Figure 12. These proposals are highly speculative, since they have been extrapolated from biochemical and physiological studies of zinc in other tissues and bioloeiral processes. Little direct evidence explicitly relates mossy fiber zinc to any of the proposed roles. Several of the hypotheses specify a close relationship between zinc and an excitatory substance, presumably a neurotransmitter or modulator. The rationale for this assumption is based on

both theoretical and experimental grounds. The concepts of neurohumoral transmission (cf. review by Gaddum, 1963) would lead to the expectation that action potentials in mossy fibers would influence CA3-4 neurons by releasing a chemical substance at synapses along pyramidal apical dendrites. Since granule axons elicit powerful excitatory effects when activated (Andersen and LoVno, 1966; Cloor et al., 1963), it seems reasonable to suspect that mossy fiber boutons contain some releasable substance which excites postsynaptic receptors. Haug and coworkers (1971) found that the zinc (or compounds containing zinc) within the mossy fibers is metabolically labile. They also argued that zinc may not be required for the short-term structural integrity of the boutons or organelles (Figure 12; a,b). These conclusions were based on the results of anterograde degeneration of mossy fibers. The Timm reactivity of the mossy fiber layer diminished rapidly after lesions in the granule cell layer; changes in the ultrastructural morphology of the boutons were not evident until much later.

Colburn and Maas (1965) stressed the importance of divalent cations in the binding, storage, and transport of putative transmitters. In particular, they proposed that magnesium, copper, iron, and zinc are present in cerebral synaptosomes and synaptic vesicles in amounts sufficient to complex with catecholamines by coordinate bonding (Maas and Colburn, 1965). Similarly, alkaline-earth metals enhance the aggregation of indolamines (Berneis et al., 1969). It seems unlikely, however, that monoamines are bound to zinc in mossy fiber terminals. Neither the granule cells nor the mossy fiber laye-exhibit the formalin histofluorescence so characteristic of the sites of norepinephrine, dopamine, or serotonin storage (Anden et al., 1966; Blackstad et al., 1967; Eidelberg et al., 1967). It is probable that the catecholaminergic and serotonergic terminals in other hippo-campal layers arise from cell bodies clustered in the brain stem. The large serotonin concentration normally present in homogenates of whole hippocampus (Paasonen et al., 1957) is markedly decreased by lesions in either the median forebrain bundle (Moore and Heller, 1967) or the raphe nuclei (Kuhar et al., 1972); these lesions interrupt major ascending monoaminergic pathways. Furthermore, the firing rates of CA3 pyramids are consistently depressed by microiontophoretic administration of norepinephrine and serotonin (cf. review, Salmoiraghi and Stefanis, 1967). For these reasons, it appears doubtful that any of the familiar biogenic monoamines serve as the excitatory transmitter in mossy fiber boutons.

Excitation of CA3 pyramids is readily elicited by iontophoretic acetylcholine (Herz and Nacimiento, 1965). However, cholinergic presynaptic elements in the hippocampus are the terminals of fibers

FIGURE 12



Stylized mossy fiber bouton illustrating several possible functions for zinc. The associations are all hypothetical, but they are based on the actions of zinc in other biological systems. The metal presumably enters the bouton by an axoplasmic route or by direct transport across the bouton membrane. Explanation of the possibilities: a. incorporation into a macromolecule of structural or catalytic function (Vallee, 1959), such as a metalloenzyme; b. membrane stabilization by aggregation around organelles (Chvapil et al., 1972), for example, vesicles containing an excitatory substance or neurotransmitter (Ex); c. transport of Ex across vesicular or synaptic membranes (Rasmussen, 1970); d binding with Ex in vesicles (Rajan et al., 1971) to stabilize storage form of the transmitter; e. ionic exchange for Ca^{++} in the initial step of transmitter release (Rubin, 1970); f. inhibition of Mg^{++} dependent $NA^{+}K^{+}$ ATPase (Donaldson, et al., 1971); g. reuptake of Ex from the extracellular to the intracellular space (Prakash, et al., 1973); h. mechanisms controlling permeability of membrane to Ex, such as inhibition of sialidase (Öhman et al., 1970).

which originate in or pass through the septum (Shute and Lewis, 1967). The enzymatic activities of choline-acetylase and acetylcholinesterase in the hippocampus are negligible after septohippo-campal pathway ablations (Lewis et al., 1967). Furthermore quantitative and histochemical studies on the distribution of acetylcholinesterase indicate the activity of the enzyme is minimal in the mossy fiber layer (Storm-Mathisen and Fonnurh, 1972).

Glutamate in the Mossy Fiber Layer

Certain acidic amino acids, especially glutamic and aspartic acids, have striking excitatory effects on the discharge rates of a variety of neurons (Curtis and Watkins, 1965; Krnjevic, 1970), including pyramidal cells (Biscoe and Straughan, 1966; Steiner, 1969). Since pyramidal cells are also excited by activating mossy fibers, the transmitter responsible for the effect might be an amino acid. If this were the case, we would expect that the concentration of the amino acid in the mossy fiber layer would be large relative to other hippocampal tissues.

With this line of reasoning, the free amino acid concentrations in the mossy fiber layer were compared to the concentrations in an analogous CM apical dendritic zone, a region devoid of zinc-rich boutons (Crawford and Connor, 1973a). Pooled samples were obtained from frozen cat brains with the aid of a dissecting microscope. The concentrations of acidic and neutral amino acids were determined with an automatic analyzer. The levels of glutamic acid and glutamine in the mossy fiber layer were about double those in the CA1 region. By contrast, aspartic acid concentrations in the two tissues were not statistically different (Table 2). Interestingly, the enzymatic activity of mossy fiber L-glutamic acid dehydrogenase (EC 1.4.1.3), a catalyst for brain glutamate synthesis (Berl et al., 1962), was about twice that of the CA1 dendritic layer (Crawford and Connor, 1973b). At least two relationships between zinc and glutamic acid which might explain their presence in mossy fiber boutons can be offered: glutamate dehydrogenase is a zinc-metalloenzyme (Adelstein and Vallee, 1958), and glutamic acid molecules form stable complexes with zinc ions by ligand bonding (Gramac-cioli, 1966). Hesitancy, on our part, in interpreting these data further is a reflection of the necessity for much additional research before a precise function can be assigned to mossy fiber zinc.

TABLE 2

Concentrations of Three Amino Acids and Activity of GDH in Two Regions of Hippocampal Neuropil [a]

Apical Dendritic Layer	Free Amino Acids [b] (nmoles/mg protein)			GDH [c] (units/mg protein)
	Aspartic	Glutamic	Glutamine	
CA1	15.3 ± 2.4	44.6 ± 5.1 ***	26.5 ± 2.3 *	2.79 ± 0.34 **
CA3	19.9 ± 0.6	102.7 ± 6.4	41.5 ± 4.5	6.54 ± 0.28

[a] Results are means ± S.E., n = 4 (except for glutamine, n = 3), protein measured by the Lowry method.

[b] Determined with a Beckman 120C amino acid analyzer after extraction (5% trichloroacetic acid), lyophilization, and resuspension (lithium-citrate buffer, pH 2.8).

[c] L-glutamic acid dehydrogenase. One unit of enzyme activity represents the synthesis of 1 μmole/hr of glutamic acid measured kinetically from the rate of NADH oxidation at 340 mμ.

* $2P < 0.050$, two-tailed, two-sample t test, CA1 vs CA3; ** $2P < 0.025$; *** $2P < 0.005$

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SUMMARY

Large concentrations of zinc in the hippocampus of many species mark this phylogenetically old area of the cortex as a potentially fruitful region for investigating the functions of a trace metal in the brain. Much of the zinc is localized within the mossy fiber layer and can be vividly demonstrated by intravital chelation with dithizone. The neurochemical and physiologic roles of mossy fiber zinc are unknown. The relationship between zinc and hippocampal function has been analyzed by: (1) correlating ontogenetic changes in hippocampal zinc with emerging cytomorphology in maturing rats, (2) studying the effect of a zinc chelator on electrophysiologically evoked waves, and (3) examining other endogenous substances which may be involved with zinc in synaptic function.

The observation that a histochemical reaction for zinc in the hippocampus of the developing rat is not present until the 20th day after birth served as a basis for studying the concentration and localization in the maturing hippocampus. Quantitative analyses by atomic absorption spectrophotometry indicated that the hippocampus was the only brain area of those examined in which zinc increased markedly (35 percent) during the 18-22 day period, to reach adult levels (2108 ± 84 p mol/mg protein). The **in vivo** uptake and localization of ^{65}Zn was studied by autoradiography. In 10 to 18-day-old rats, 72 percent of the positron tracks counted were in the granule cell layer. After 20 days of age, most tracks were noted in the mossy fiber layer. The changes in localization and concentration of zinc may be related to granule cell morphogenesis and functional ontogeny. The sudden appearance of zinc in the mossy fibers could, in turn, influence developing behavioral patterns in young rats.

The selective intravital affinity of dithizone for mossy fiber boutons provides a unique approach for determining the effects of zinc chelation on mossy fiber function. Alterations of electrophysiological waves after systemic doses of dithizone were studied in unanesthetized adult male rats with electrodes implanted chronically to record from the hippocampus. Low doses (25 mg/kg, i.p.) potentiated the response evoked by entorhinal stimulation (mossy fiber activation), whereas higher doses (100 mg/kg) blocked the wave. The drug had little effect on waves evoked by commissural stimulation. Frequent administration (25 mg/kg/hr, i.p., 12 hour) resulted in two major physiological effects. The potentiated response usually seen after repetitive entorhinal stimuli was absent; single stimuli readily produced after-discharges, often accompanied by local seizure activity. Lessened histochemical reactivity suggested depletion of zinc from the mossy fiber pathway; by quantitative analyses, hippocampal zinc was reduced by 20 percent. These results indicate that zinc may be required for activating pyramidal cells through the mossy fiber pathway. By virtue of its limbic connections, the hippocampus influences many brain activities; thus changes in the concentration or availability of zinc in mossy fibers might contribute to a variety of cerebral physiopathologies.

The axons of the granule cells form powerful excitatory synapses with the apical dendrites of CA3-4 pyramidal cells. Since certain amino acids are putative excitatory neurotransmitters, we measured the free acidic amino acids in samples microdissected from various pyramidal cell layers. Relative to other layers, a large amount of glutamic acid was present in samples rich in CA3 apical dendrites. We also compared the enzymatic activity of L-glutamic acid dehydrogenase, a zinc metalloenzyme. Activity in the mossy fiber layer was more than twice that of the CA1 apical dendritic layer. Thus, a synthetic enzyme and its excitatory amino acid were located in a hippocampal layer which also contains notable concentrations of zinc. In conclusion, the sum of the available evidence suggests that zinc is important in the ontogeny of hippocampal function

and that the trace metal may subserve synaptic functions in neurotransmission.

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