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OCCURRENCE OF MANGANESE, COPPER AND ZINC IN MYELIN. ALTERATIONS IN THE PERIPHERAL NERVOUS SYSTEM OF DYSMYELINATING TREMBLER MUTANT ARE AT VARIANCE WITH BRAIN MUTANTS (QUAKING AND SHIVERER)

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Abstract—Mn, Cu and Zn were present in mouse brain at concentrations that were 54, 4 and 14 times higher than in serum. In comparison with control animals, Mn was nearly normal in both quaking and shiverer dysmyelinating mutants. Cu was slightly higher in shiverer; Zn was higher in quaking only when expressed on a dry weight basis.

The peripheral nervous system contained lower amounts of Mn, Cu and Zn than brain, (1/6, 1/8 and 1/2 respectively). All three metals were much higher in trembler (4, 3 and 2-fold increase, respectively). Although higher in shiverer and quaking, Mn did not differ significantly from control. Cu and Zn were similar to control in the sciatic nerve of quaking and shiverer.

Brain myelin contained Mn, Cu and Zn concentrations that were slightly smaller than those found in the whole brain. Mn and Cu were higher in the myelin from shiverer by approx. 2- and 3-times, whereas Zn was two-fold reduced.

It is speculated that such metals play a role in membrane as cofactors of enzymes, especially those in control of free radical damage, and possibly also in membrane structures as phospholipid counterions.

The brain is vulnerable to either a deficit or an excess of available trace elements. An impaired metabolism of trace elements manifests itself with particular intensity in the brain, with effects on both the formation and integrity of myelin. However the concentration of Mn, Cu and Zn in myelin, in neurological dysmyelinating mutants and in the peripheral nervous system has not been documented.

Manganese

The manifestations of manganese deficiency reveals widespread neuronal degeneration and glial proliferation. The symptoms and signs of manganese encephalopathy share several features in common with Parkinson's disease and there is a direct link between the metabolism of catecholamines and manganese concentrations in the brain (Chandra, 1983).

This metal is essential for many enzymes: hydrolases, kinases, decarboxylases and transferases. Manganese and magnesium are important metal ions associated with ATP, and manganese can replace magnesium in several biological reactions of the metal-ATP complex, including adenylate-cyclase.

Manganese deficiency and intoxication seem to alter myelination only to a slight extent. However, some myelin enzymes are stimulated by Mn, e.g. 5′nucleotidase (Cammer *et al.*, 1980) and phosphoprotein phosphatase (Yourist *et al.*, 1978). The myelin marker CNPase has been reported to be inhibited by 20% by an excess of Mn (Sprinkle and Knerr, 1981).

Copper

The brain is second only to the liver for its concentration of copper on a dry weight basis. The brain is vulnerable to either a deficit or an excess of available copper (Smith, 1983). The most consistent and readily demonstrated effects of copper deficiency on the developing brain are those on myelin or on the

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process of myelination, but the molecular basis of such effects remains unclear in animal (Zimmerman *et al.*, 1976; Prohaska J. R. and Wells W. W., 1974; Di Paolo *et al.*, 1974; Everson *et al.*, 1978; Smith *et al.*, 1977; Barlow *et al.*, 1960; Barlow, 1969) and in Menkes' disease in human infants. In human Wilson disease, a mutation causing accumulation of copper in brain rather than a deficiency as in Menkes disease, neurological consequences are important (Sass-Kortsak and Bearn, 1978; Smith *et al.*, 1981).

The metabolic studies of copper in developing rat brain show changes in time consistent first with the development of cytochrome oxidase activity and secondly with the appearance of dopamine-beta-hydroxylase and superoxide dismutase in the cytosol (and possibly in myelin) (Smith, 1983). Moreover the various roles of copper in brain development are related to the metabolism of oxygen.

Zinc

Zinc is essential for the growth and maintenance of many cellular components (Halas, 1983; Crawford, 1983). It is a constituent of several metalloenzymes, and its role in protein metabolism and nucleic acid synthesis is only partially understood. A possible role for the Zn-enzyme in developing brain has been proposed in connection with maturation and myelination (Cohn and Richter, 1956; Chvapil, 1976). The activity of the enzyme 2′, 3′-cyclic nucleotide 3′-phosphohydrolase (CNPase: EC 3.1.4.37) was found to be significantly reduced with zinc deficiency in the brain (Dreosti, 1981).

Neurological mutants

Trace elements (Cu and Zn) have been only analysed in quaking brain, and contradictory results were obtained.

Keen and Hurley (1976) reported that copper content was reduced in the brains of 21-day-old quaking mice on the C_{57} BI/6J background whereas copper is not reduced in nonneural tissue (Hurley *et al.*, 1980). After dietary supplementation in copper, copper concentrations were increased and the characteristic body tremors were reduced (Keen and Hurley, 1976). Prohaska (1980) found no deficit in brain copper concentration in adult quaking animals of the same genetic background.

Rosenfeld *et al.* (1983) found that brain copper was increased by 84% above normal mice at the 21st day after birth, but was not significantly different from normal in adults. Zinc was increased by 23 and 24% in young and adult quaking, respectively.

Reviews on myeline (Morell, 1984) and on dysmyelinating mutants (Baumann, 1980; Hogan and Greenfield, 1984) have been recently published.

This work was undertaken to: (i) to determine if Mn, Cu and Zn were present in the peripheral nervous system in control and dysmyelinating Trembler mutants; (ii) to determine if Mn, Cu and Zn were present in myelin; (iii) measure alteration of Mn, Cu and Zn in brain of neurological mutants, quaking and shiverer;.

Results have been presented in abstract form (Bourre *et al.*, 1986).

EXPERIMENTAL PROCEDURES

Trembler mutant was on B6-CBA strain and originate from the scottish mutation (through laboratories of Dr Guenet, Pasteur Institute); Shiverer, on C3H-SWV, was obtained from Washington University (Dr Bird). The quaking was on C57–B6 black and originated from Jackson laboratories. These mutants were kindly provided by Dr Baumann (INSERM U-134) and bred in our laboratory.

Animals were compared with their normal appearing littermate, except for shiverer which were compared with a control strain.

Brain myelin was prepared according to Norton and Poduslo (1973) as routinely done in our laboratory. The purity was checked by electron microscopy, marker enzymes, protein analysis and lipid analysis as published in our preceding papers (Bourre *et al.*, 1982, 1984).

Blood was collected by heart puncture.

Tissue was lyophilized and weighed, digested in pure nitric acid (300 μl, d = 1.40) at 100°C for 17 h in quartz tubes, completed to 5 ml with bidistilled deionized water. After dilution, aliquots were injected.

Mn was determined by flameless atomic absorption (Varian AA5) and inductive conductor plasma (ICP, IPOS 1500). Cu and Zn were determined by flameless atomic absorption. For flameless determination, Internal method was used. For plasma, standard curve with metals were used.

Chemicals were from Sigma and Merck. Water was deionized and bi-distilled. We checked that all solutions were totally free of trace elements. Moreover dissolved tubes and glass-ware did not contain significant amounts of trace elements. Only scissors treated for 24 h in the presence of acid released trace amount of Mn.

Statistical analysis was performed using the Student's *t*-test.

RESULTS AND DISCUSSION

Trace elements in peripheral nervous system (Table 1)

Substantial amounts of Mn, Cu and Zn were persent in the peripheral nervous system. However, these trace elements were much less concentrated than in the brain, approx. 6 and 8 times less for Mn and Cu, and two-times less for Zn.

Shiverer and quaking sciatic nerve contained normal amounts of Cu and Zn. In Shi and Qk, Mn was

Table 1. Mn, Cu and Zn in control and trembler sciatic nerve

	Mn (nmol/g)		Cu (nmol/g)		Zn (nmol/g)	
	Dry	Wet	Dry	Wet	Dry	Wet
Control (16)	5.1	1.6	35	11	550	177
Trembler (7)	20.8***	4.3***	110***	23***	1300***	266***
Shiverer (6)	7.6^{ns}	2.3^{ns}	32^{ns}	10^{ns}	750^{ns}	232^{ns}
Quaking (3)	7.6^{ns}	2.4^{ns}	24^{ns}	9^{ns}	500^{ns}	158^{ns}

Mn, Cu and Zn in sciatic nerve from control, trembler, shiverer and quaking mice. Numbers in parentheses represent the numbers of pooled nerves, and thus the number of determinations. Each pool was made with 6 animals. A total of 96, 42, 36 and 18 animals was used for control, trembler, shiverer and quaking, respectively. ns = non significant; *** = $P < 0.001$.

higher than in control, but this difference was not significant.

In the trembler, Mn, Cu and Zn were dramatically increased: 4-, 3- and 2-times, respectively. In this mutant the amount of myelin is drastically reduced, and Schwann cells were largely proliferating. Thus this increase in trace element could be related to the relative increase in Schwann cell plasma membrane or to the dramatic increase in basal laminae. Trembler Schwann mitochondria seems to be increased by 70% and hypertrophied (Low, 1976a, b). Thus Mn could be related to increased mitochondrial Mn-superoxide dismutase in trembler. Moreover, the abundant collagen could bind the metals ions also in trembler.

Trace elements in brain myelin (*Table* 2)

Brain myelin contained measurable amounts of Mn, Cu and Zn. Based upon dry weight, these trace elements were slightly less concentrated in myelin than in the whole brain. Interestingly, in the myelin of shiverer brain Mn and Cu were increased by 2 and 3 times respectively, whereas Zn was reduced by half.

Table 2. Mn, Cu and Zn in brain myelin

	Mn (nmol/g dry)	Cu (nmol/g dry)	Zn (nmol/g dry)
Control			
0.85 M (11)	23.2	233	610
0.9 M (3)	22.0	200	1000
Shiverer (4)	41.3***	650***	320***

Mn, Cu and Zn in normal and shiverer brain myelin. Numbers in parentheses represent the number of myelin preparation. Trace elements were measured on each. One myelin preparation required 9 animals for control, and 32 for shiverer (a total of 99 animals was used for control 0.85 M myelin, 27 for 0.9 M myelin, 128 for shiverer myelin). Shiverer myelin was prepared on 0.9 M sucrose as we have previously shown that the abnormal myelin in this mutant was immobilized on 0.9 M sucrose using a continuous gradient (Bourre *et al.*, 1980). In control, myelin was also eventually prepared on 0.9 M sucrose to decide if the increase in Mn and Cu in shiverer myelin was not due to contamination by a 0.85-0.9 M fraction that could be rich in these trace elements. ***: $P < 0.001$.

These trace elements may be related to unknown enzyme activities, besides known ones: presence of Mn-sensitive 5′-Nucleotidase (Cammer *et al.*, 1980; Bourre *et al.*, 1982) Zn carbonic anhydrase (Cammer *et al.*, 1976), and Cu-Zn superoxyde dismutase (Thomas, 1976; Loomis and Stahl, 1976).

Trace elements in membrane could play a role in the structure of the bilayer. Mn-lipid associations could be responsible for phospholipids compartmentation in microsomes. (Binaglia *et al.*, 1985). Thus another explanation for the asymmetric distribution of the membrane lipids seems of particular interest: besides membrane proteins which could create lipid domains and restrict the mobility of some lipid molecules, the phospholipid counterions could play a relevant role in maintaining a specific membrane topography.

Zn modifies the interaction between myelin membrane in a unique way through molecular interaction between zinc, glycoproteins and lipids (Inouye and Kirschner, 1984). Zn is capable of preventing peroxidation of liver membrane lipids and has been shown to have a stabilizing effect on lysosomal membranes (Chvapil, 1976; Bettger *et al.*, 1978).

A specific role in free radicals scavenging and protection of polyunsaturated fatty acids in membrane may be proposed for trace elements.

Zn deficiency alters the fluidity of microsomal membranes from liver, testes and the small intestine. Zn and Cu appear to have opposite effects on lipid peroxidation and membrane stability. Manganese significantly inhibits the ability of treated rat brain to undergo lipid peroxidation, without altering the contents in iron and ascorbic acid, two prooxidant factors when present together (Shukle and Chandra, 1981; Donaldson *et al.*, 1982). Thus manganese, like cobalt and zinc, inhibit the formation of lipid peroxides. The binding of manganese with membrane phospholipids could provide protection against the oxidation.

Table 3. Mn, Cu and Zn in brain and blood of control, quaking and shiverer mice

	Mn (nmol/g)		Cu (nmol/g)		Zn (nmol/g)	
	Dry	Wet	Dry	Wet	Dry	Wet
Control (21)	30.6	6.3	290	61	1180	246
Quaking (17)	34.4*	7.1*	290^{ns}	56^{ns}	1430***	253^{ns}
Control (15)	31.1	6.3	291	53	1105	224
Shiverer (14)	38.6*	7.3*	349**	66**	1180^{ns}	280^{ns}
Serum (nmol/ml)						
Mouse (10)	0.118		14.6		18.0	
Human (15)	0.019		19.4		20.3	

One measurement was performed for each brain. The figure number in parentheses gives the number of brains that were used. ns: non significant; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$.

Trace elements in brain (*Table* 3)

Manganese. The measured manganese concentration in the whole brain of mouse (1.69 μg/g dry weight) was in agreement with results obtained in rats: 1.73, mean values from 5 papers, and even in humans (1.8, mean value from 7 papers for various anatomical regions). On a dry weight basis, Mn was increased by 12% in quaking and by 24% in shiverer. On a wet weight, Mn was increased by 12% in quaking and by 16% in shiverer. Alterations observed on a wet weight basis were poorly related to the reduced amount of solid in both mutants, or to the increased quantity of water: dry weight/wet weight was 0.205 in control, 0.173 in quaking, and 0.189 in shiverer ($P < 0.001$ for both quaking and shiverer in comparison with control).

Copper. Our value for copper in normal brain (290 nmole/g dry weight, 18.3 μg/g dry weight) was in agreement with previously published data: 16.2 μg/g (Rosenfeld *et al.*, 1983) 14 μg/g (Terao and Owen, 1977), 12.0 μg/g (Smith 1983); 23 and 29 μg/g in human (mean values, Smith 1983; Goldberg and Allen, 1981).

Copper was normal in quaking brain when related to wet weight and dry weight. In shiverer, copper was increased by 20% related to dry weight and by 25% related to wet weight. As myelin contained a lower concentration of copper than the whole brain, this increase in the shiverer could be due to the nearly absence of myelin in this mutant. However this was not observed in quaking, another mutant also nearly devoid of myelin.

Copper was concentrated in the brain as the concentration is approx. 4 times higher than in the blood.

Our results in quaking were in agreement with Rosenfeld *et al.* (1983) showing that copper is not affected in adult animals, based upon dry or wet weight. However quaking mice had a significantly reduced content of solids ($P < 0.001$) indicating increased water content in the brain of mutants.

Zinc. Zinc concentration in the normal brain is this study compared favorably with previously published results [77.1 μg/g dry weight in this study, 78.1 by Rosenfeld *et al.* (1983) in rat; 49 μg/g by Butt *et al.* (1956) and a mean value of 60 μg/g by Donaldson *et al.* (1972) in human].

For quaking we were in agreement with Rosenfeld *et al.* (1983). Zinc was increased by approx 20% when related to the dry weight, but normal if related to wet weight in adult animal. Zinc was normal in shiverer.

Mn, Cu, Zn were present in brain at concentration 54, 4 and 14 times higher, respectively than in serum (the concentrations of Cu or Zn were similar in mouse and human, whereas Mn was more concentrated in mouse serum).

This work provides original data on Mn, Cu and Zn in myelin, in the peripheral nervous system, in the brain of shiverer and in the brain of quaking mutants.

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