Regional Metal Concentrations in Parkinson's Disease, Other Chronic Neurological Diseases, and Control Brains

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ABSTRACT: Metal deficiency or toxicity states have been recognized as a cause of several neurological disorders and are suspected in others. We analyzed four brain regions (frontal cortex, caudate nucleus, substantia nigra, and cerebellum) in 36 human brains for concentrations of 24 metals (Ag, Al, As, B, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Pb, Mg, Mn, Mo, Na, Ni, P, Se, Ti, V, W, Zn). Regional metal concentrations, measured using atomic absorption and atomic emission spectroscopy, were compared between 9 Parkinson's disease (PD) brains, 15 brains from patients with other chronic neurological diseases, and 12 control brains. No significant metal concentration differences were noted between brains from PD and other chronic neurologic disease. However, parkinsonian brains (PD and parkinsonism secondary to neurofibrillary tangle disease) showed lower concentrations of magnesium in the caudate nucleus and copper in the substantia nigra than control brains. These findings may represent an etiologically important clue to parkinsonism.

RÉSUMÉ: Concentrations régionales de métaux dans le cerveau de patients souffrant de la maladie de parkinson, d'autres maladies neurologiques chroniques et de sujets contrôles Un déficit ou une surcharge en métaux est reconnue comme étant la cause de plusieurs affections neurologiques et l'on soupçonne ce mécanisme d'être à l'origine de certains autres désordres neurologiques. Nous avons analysé la concentration de 24 métaux (Ag, Al, As, B, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Pb, Mg, Mn, Mo, Na, Ni, P, Se, Ti, V, W, Zn) dans quatre régions du cerveau (cortex frontal, noyau caudé, substance noire et cervelet) de 36 sujets. Nous avons comparé les concentrations régionales de métaux dans le cerveau de 9 patients atteints de la maladie de Parkinson, 15 patients présentant d'autres maladies neurologiques chroniques et 12 sujets contrôles. La détermination des concentrations de métaux a été effectuée par spectroscopie d'absorption atomique et par spectroscopie d'émission atomique. Nous n'avons pas trouvé de différence significative entre les concentrations de ces métaux dans les cerveaux des patients atteints de la maladie de Parkinson et ceux des patients atteints d'autres maladies neurologiques chroniques. Cependant, les cerveaux des parkinsoniens (maladie de Parkinson et parkinsonisme secondaire à la maladie des amas neurofibrillaires) contenaient des concentrations plus faibles de magnésium au niveau du noyau caudé et de cuivre au niveau de la substance noire que les cerveaux des sujets contrôles. Ces observations peuvent constituer un indice important quant à l'étiologie du parkinsonisme.

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The cause of Parkinson's disease (PD) is not known. However, most investigators believe that environmental factors play an important role in the etiology.[1,2] The search for toxic compounds potentially responsible for PD has been accelerated with the recent discovery of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) induced parkinsonism.[3,4] This study represents another avenue of the broadening search for the cause of PD.

Metals are essential cofactors in certain enzymatic reactions and important components in many human proteins and enzymes.[5] Metal deficiency or toxicity states have been recognized as a cause for several genetic or acquired neurological disorders. For example, defective copper metabolism is the basis of Wilson's disease, a hereditary disorder resulting in excess copper deposits in various organs which manifests primarily as an extrapyramidal syndrome. On the other hand, copper deficiency, in Menkes' disease, an X-linked disorder, results in psychomotor retardation, seizures, coarse brittle hair, and premature death.[6] Toxicity due to other metals, resulting in neurological dysfunction, may also be acquired by an otherwise healthy individual, e.g. Minamata disease due to methyl mercury toxicity,[7] lead toxicity,[8] and parkinsonian syndrome due to chronic manganese toxicity.[9]

Metal toxicity states may occur in unsuspected circumstances. Recently, exposure to the fungicide maneb has been associated with the signs and symptoms of manganese poisoning and a parkinsonian syndrome.[10] Altogether, nine different metals: aluminum, arsenic, calcium, copper, iron, manganese, magnesium, mercury, and zinc have been suspected in the etiology of parkinson syndrome.[5]

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While there are several reports dealing with concentrations of metals in human brains with neurological diseases and in normal brains, most of these studies have been limited to small numbers of cases and have measured single or only several metals at a time.[11,12] As well, the relative concentration of one metal may influence the concentration and action of another metal, e.g. zinc and copper,[13,14] and magnesium and calcium,[15] are known to interact metabolically. Hence, the significance of a small number of metal concentrations in the pathogenesis of a given disorder is difficult to interpret. We decided to study four anatomical brain areas in 9 PD brains, 15 brains from a variety of chronic neurological diseases and 12 control brains, measuring simultaneous concentrations of 24 metals to identify any abnormalities in metal concentration specific to PD. Included in the chronic neurological disease group were several brains from patients who had suffered from parkinsonism on the basis of neurofibrillary tangle diseases rather than Lewy body disease.

Material and Methods

Brain tissue samples, as a rule, were obtained from the left hemisphere. Where the left side was diseased in a manner which might interfere with chemical analysis, e.g., a recent stroke or tumor, the specimen was obtained from the right side. Moreover, only those brains where an unequivocal histological diagnosis had been made by a qualified neuropathologist (BR) were sampled. The neurological disorders included nine cases of Lewy body Parkinson's disease (PD) (M-6, F-3, mean age 73 yrs.), four of motor neuron disease (MND) (M-2, F-2, mean age 63 yrs.), four of senile parkinsonism (SP; widespread neurofibrillary tangle disease with parkinsonism; M-2, F-2, mean age 75 yrs.), two parkinsonian cases without Lewy body disease but with abundant neurofibrillary tangles limited to the substantia nigra and locus ceruleus (NFT) (M-1, F-1, mean age 75 yrs.), two Alzheimer's disease (AD) (M-1, F-1, mean age 70 yrs.), one multiple sclerosis (MS) (F-1, age 72 yrs.), one Huntington's disease (HD) (F-1, age 66 yrs.), and one Down's syndrome and Alzheimer's disease (DSAD) (M-1, age 66 yrs.). The twelve control brains (M-8, F-4, mean age 70 yrs.), included five cases that had no clinical or pathological evidence of neurological disease, one that showed some demyelinating changes in the spinal cord only, three with unilateral cerebral tumor, and three with unilateral acute cerebral infarct. In the control cases, the pathologically unaffected hemisphere was used for the analysis.

The method utilized for metal analysis was identical to that previously reported.[16] All brains were fixed with buffered 10% formalin within 24 hours after death. Samples from four brain areas — parasagittal frontal cerebral cortex (FC), the central portion of caudate nucleus (CN), zona compacta and reticularis of the substantia nigra (SN), and cortex and subcortical white matter of cerebellum (CB) — were utilized for analysis in both the cases and the controls. The chemist responsible for analysis (WKY) was blinded to the diagnosis and the source of the tissue specimens. Samples from formalin-fixed brain were kept frozen (−15° C) until preparations were made for the chemical analysis, and the sample tissue was then dried at 105° C and weighed. The weight of specimens ranged from 50 to 500 mg. Occasionally, clean, stainless steel scalpels were used to slice large specimens prior to dissolution. Tissue samples were each dissolved initially in 10 ml of nitric acid, with an additional 3 ml of nitric acid and 3 ml of perchloric acid being added prior to application of heat, which continued until evolution of perchloric fumes and complete tissue dissolution. The solution was then transferred to a 50 ml volumetric flask and taken to volume with deionized water. Acid blanks underwent an identical procedure. Using instruments calibrated with low concentration metal standards, atomic emission spectroscopy and atomic absorption spectroscopic measurements were carried out. Tissue solutions were analyzed for aluminum (Al), arsenic (As), beryllium (Be), boron (B), cadmium (Cd), calcium (Ca), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), phosphorus (P), potassium (K), selenium (Se), silver (Ag), sodium (Na), titanium (Ti), tungsten (W), vanadium (V), and zinc (Zn). Concentrations of metals in the tissue samples were calculated using dry tissue weights in units of μg/g dry weight. Mean and standard error of the mean were calculated for each metal-brain area.

Nitric acid (70%) and perchloric acid (70%) were of ACS grade. Buffered 10% formalin for fixation contained 4 g $NaPO_4$ and 6.5 g $NaPO_4$ per liter. Instrument calibration standards (1000 ppm aqueous solution of individual elements) were from the British Drug House (BDH) Canada. A Jarrell-Ash Model 1140 ICP Spectrometer (inductively coupled plasma) was used for metal determinations, except for As and Se. The latter two were determined through the use of a Perkin-Elmer 503 atomic absorption spectrophotometer and a MH-10 hydride generator. These instruments were calibrated with low concentration solutions prepared by serial dilutions of 100 ppm standards. A Standard Reference Material (SRM) from the National Bureau of Standards (Washington, D.C.) was used as a quality control check to assure metal concentration measurements were accurate; bovine liver (SRM#1577) has certified values for the following elements: As, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Pb, Se, and Zn. Not all metals were detected in each brain area analyzed.

Statistical analysis was performed by a qualified statistician (MB). The data were transformed to normal scores prior to analysis with scores for tied values averaged. This procedure attempted to accommodate the skewed distribution of the data, as well as the large number of results that were below detection limits.

Multivariate statistical methods, on a brain-region-specific basis, were applied only to those metal variables in which data were available from every patient. These included 15 metals in the caudate nucleus (Ag, Al, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Ti, and V) and 12 in the cerebellum (Ag, Al, Ca, Cd, Cr, Cu, Fe, Mn, Na, Ni, and Ti). In addition, to allow for the possibility that total metal levels (non-specific to a particular brain region) might prove most indicative, the mean level of each metal averaged over all four brain regions was also analyzed. After excluding metals which were undetected in most patients, this analysis included 11 metals (Al, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, and P). Multivariate analysis of variance (MANOVA) was performed on both the region-specific and region-averaged variables. In addition, dimensional reduction was attempted by transforming the region-specific data to principal components of the total correlation matrix,[17] and performing the MANOVA on the principal components explaining 95%

of the variation. Univariate analyses were also performed on the variables marginally, adjusting for multiplicity by the Bonferroni correction.[18] Significance was assessed by the likelihood ratio criterion (Wilks' lambda), using Rao's F-approximation of the distribution.[19] All data analysis was performed using SAS 5.03 (SAS Institute, 1985), installed on a VAX/VMS system, at the University of Saskatchewan, Saskatoon, Saskatchewan.

Results

Mean concentrations and the standard errors of the mean for metals measured in all PD brains, other disease groups, and control brains were calculated. Results for PD brains and control brains concerning eight of the nine metals implicated in parkinsonism are shown in Table 1. (A complete listing of all brain metal concentrations for each pathologic entity is available from the authors on request.)

The MANOVA does not indicate any significant differences between the groups, either on a region-specific ($p = 0.261$) or region-averaged ($p = 0.546$) basis. The attempt at dimensional reduction is not successful, requiring 15 principal components to capture 95% of the variation. MANOVA of these components also does not show any significant difference ($p = 0.694$). The comparison of controls with all forms of parkinsonian patients (PD, SP, and NFT) taken together, also yields no significance ($p = 0.332$).

Seventy-six variables give above-threshold levels for some patients. Two of these variables, Co in the substantia nigra in the only Huntington's disease brain and V in the caudate nucleus of one PD brain, are found in only single specimens and are probably of only anecdotal value. The other 74 variables give p-values ranging from 0.022 to 0.997 for the hypothesis of no group differences. None of these variables approach the Bonferroni limit of 0.05/74 = 0.007 required to establish 5% significance. The distribution of p-values comes close to what one would expect from a random sample of independent variables having no differences among the groups. However, the two most significant variables are Cr levels in the SN and FC. These appear to average lower in the control group than in the non-parkinsonian disease groups. The analysis of the region-averaged data also suggests the significance of Cr ($p = 0.016$), although some of the patients with the highest Cr levels in CN and CB are controls.

Restricting analysis to a comparison of the control group with the three parkinsonian sub-groups (PD, SP, and NFT), a somewhat different picture emerges. Here the two most determinative variables are magnesium in the caudate nucleus ($p = 0.0019$) and copper in the substantia nigra ($p = 0.0067$). These metals were, on average, present at lower levels in the parkinsonian patients than in controls. Although these values remain above the Bonferroni bound required for 5% significance, the fact that two P-values were below 0.01, even out of 74 variables, gives some credibility to the possibility that these observations are not spurious.

Table 1: Mean Metal Concentration by Brain Region in PD and Control Brains

Metal		FC		CN		SN		CB	
		PD	Control	PD	Control	PD	Control	PD	Control
Al	x	5.0	5.2	2.9	10.7	10.2	7.7	1.4	7.9
	s_x	0.8	1.3	1.1	4.1	3.0	2.5	0.5	4.0
As	x	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.1
	s_x	0.03	0.2	0.03	0.02	0.04	0.02	0.01	0.01
Ca	x	506	554	468	630	468	623	410	618
	s_x	50	76	43	127	62	130	35	112
Cu	x	25.0	26.5	25.7	28.6	29.3	38.8	34.1	33.0
	s_x	2.0	1.9	2.5	1.9	2.7	3.3	1.5	2.1
Fe	x	280	295	610	651	653	613	268	325
	s_x	8	21	97	44	55	56	21	30
Mg	x	482	539	471	530	414	452	515	560
	s_x	38	14	28	11	32	25	30	30
Mn	x	2.6	1.9	6.6	3.6	6.8	3.0	3.0	3.1
	s_x	0.5	0.5	3.1	1.4	4.9	0.8	1.0	1.2
Zn	x	63.0	51.2	72.5	71.0	68.8	78.6	64.8	76.9
	s_x	7.3	10.2	9.3	11.1	7.5	22.5	6.2	10.5

FC = frontal cortex CN = caudate nucleus SN = substantia nigra
CB = cerebellum PD = idiopathic Parkinson's disease (n = 9)
Control (n = 12)
x = mean s_x = standard error of mean x and s_x in ug/g dry weight

(Only eight metal concentrations are listed because of space limitation. Complete results are available from the authors upon request.)

Discussion

The four brain areas selected for metal concentration analysis are regions primarily involved in the modulation of motor activity. These areas are components of the extrapyramidal system and lesions therein are known to cause abnormalities in movement. The pre-motor area of cerebral cortex is involved in the production of complex patterned movements and the caudate nucleus is thought to be important in inhibition of spontaneous movement. The function of the substantia nigra is still poorly understood, but, it is known to have profuse connections with other areas of the brain concerned with motor control and lesions in the substantia nigra produce parkinsonism. Finally, the cerebellum is instrumental in the coordination of voluntary movements such as those required for normal motor action and gait.

Human tissue metal concentrations may be determined by a variety of methods. We chose atomic absorption/emission spectroscopy in preference to x-ray detection/fluorescence methods because the former are more sensitive in detecting lower concentration levels for most of the metals studied. A neutron activation procedure would yield comparable sensitivity and accuracy to the method we utilized, but would not permit the same tissue to be analyzed successively for multiple metals. Atomic absorption/emission spectroscopy was, therefore, deemed to offer the best combination of sensitivity/quantification and ability to simultaneously measure 24 metals in each selected tissue sample.

We were concerned about potential metal concentration changes that might occur with formalin fixation. Measuring metal concentrations in fresh/frozen brain specimens would have been ideal, but such an experiment was not feasible. Histological diagnosis, which is crucial for different pathological entities, required one-half of the brain to be fixed in formalin in most cases. Excluding the histological examination would be such a major omission as to preclude any firm conclusions being made about brain metal concentrations in a given condi-

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tion (as the diagnosis would be based solely on the clinical impression). By utilizing histologically normal brains, preserved and processed identically to diseased brains, we have, to a large extent, excluded the bias of selective metal concentration changes due to formalin fixation in diseased brains. Our method is more practical for future studies, as obtaining a large number of frozen brains is an extremely difficult proposition. No comprehensive study of metals has been reported (analyzing fresh/frozen brains or formalin preserved brains) in which as many pathologic conditions have been analyzed as in the present experiment. A standard reference material (bovine liver tissue — SRM#1577) from the National Bureau of Standards in Washington, D.C., which has certified metal concentration values, was analyzed in an identical fashion and found to contain the stated reference metal concentrations as measured by our methods, indicating that the metal concentrations reported in this paper represent accurate values.

The nature of this study necessarily produced a large pool of data on relatively few individuals, which required careful scrutiny in an effort to avoid over- or under-interpretation of all measurements, and thus the expertise of a qualified biostatistician (MB). Whereas multivariate methods may prove insensitive (or indeed impossible) in such a situation, marginal analyses of each separate variable can leave one open to the trap of "data dredging". Thus, valid and efficient analysis necessitates either a dimensional reduction technique or some suitable adjustment for multiplicity.

In an earlier report we identified significant regional differences in four areas of the brain and an age-related reduction in the concentrations of several metals in normal human brain.[16] We noted that PD brains had lower concentrations of copper in the substantia nigra and of magnesium in the caudate nucleus and cerebellum compared to normal age- and sex-matched control brains.[20] Since these were new observations and considered only PD compared to control brains, we decided to study other neurological diseases to determine if the observations were specific for PD. In this study, we have identified a possible correlation between lower levels of magnesium in the caudate nucleus and copper in the substantia nigra, and parkinson syndrome in general. These changes, therefore, cannot be regarded as specific for PD, but are common to the forms of parkinsonism in our study. We measured metal concentrations of eight of the nine metals implicated in the etiology of PD and found no significantly different concentrations in the PD brains; only one implicated metal, mercury, was not measured in this series.

Hypomagnesemia was first implicated in human parkinsonism by Poryali in 1960[21] and again by Barbeau et al.[22] Those observations, however, could not be confirmed by Schwab et al.[23] Barbeau later fed mongrel dogs a magnesium deficient diet and noted that, during the fourth and fifth weeks of administration, the animals exhibited marked hypokinesia and limb ataxia, when significant hypomagnesemia and hypocalcemia were present.[24] These clinical manifestations had some, albeit atypical, features of parkinsonism. At the end of 35 days the chemical analyses of brains from these dogs were compared with those fed a normal diet. A significant reduction in striatal dopamine concentration was noted in the dogs fed a magnesium deficient diet. This observation is similar to that seen in human parkinsonism. Such a commonality suggests that magnesium deficiency may play a role in the production of parkinson syndrome. Our data show lower magnesium levels in the caudate nucleus in all forms of parkinson syndromes including PD, indicating that magnesium deficiency is most likely not the cause of PD but rather found in association with parkinson syndrome in general.

Copper toxicity has been studied in several diseases, including parkinsonism. Chitre and Punekar reported that both serum copper levels and copper oxidase activity are significantly higher in chronic human parkinsonism.[25] They conjectured that cell degeneration within the substantia nigra of parkinsonian cases may be due to toxic levels of copper deposition within those cells which interfere with vital enzymatic systems. In untreated PD patients, significantly higher concentrations of copper in cerebrospinal fluid (CSF) have been reported.[26] Because no corresponding increase in iron or manganese was noted, the authors concluded that the copper increase did not merely represent non-specific cellular leakage into the CSF. They postulated that the elevation of CSF copper enhanced production of hydroxyl free radicals which might cause neuronal damage.

Other reports, however, suggest that copper deficiency may be crucial in parkinsonism. O'Dell and Prohaska studied dietary copper deficient ataxic lambs.[27] Dopamine levels in the striatum proved to be significantly lower in untreated copper-deficient animals. The debility in these animals improved with copper therapy. These authors speculated that copper deficiency inhibits tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. Copper is also known to be necessary for the antioxidant activity of superoxide dismutase and therefore deficiency of copper might result in difficulty clearing harmful free radicals. The activities of both tyrosine hydroxylase and superoxide dismutase have been found to be reduced in copper deficient rat brain, further strengthening the copper deficiency hypothesis.[28] We have noted lower copper levels in the SN in parkinsonism and thus our data support a possible role for copper deficiency in this disorder. Weiner et al have noted that long-term use of levodopa-carbidopa, bromocriptine, or lergotrile produce significantly increased manganese concentrations in all brain areas and decreased copper concentrations in guinea pig brain.[29] There is no comparable study in human parkinsonian patients treated similarly. It should be noted that not all of our patients received these drugs. Hence, the significance of drug therapy on copper levels in brain is not clear at this point. We can only conclude that we have found significantly lower concentrations of copper within the substantia nigra of parkinsonian (PD, SP, and NFT) brains in comparison to controls and other selected chronic neurological disease groups. Whether this finding is directly related to the disease, reflects a consequence of treatment, represents an epiphenomenon of the condition, or is merely a fortuitous aspect of our set of patients, is still open to debate. Further studies are needed to verify our observations and clarify their role in parkinsonism.

Drayer et al, using high field strength magnetic resonance imaging techniques, recently reported an increased concentration of iron in the substantia nigra of PD patients.[30] Our data, based on direct measurements from brain tissue, do not support this observation.

In summary, no role for any of the 24 metals measured was identified as specific for PD on the basis of brain regional metal

concentrations. However, our data suggest a correlation between copper and magnesium deficiency and parkinsonism.

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