Elevated striatal levels of glial fibrillary acidic protein associated with neuropathology in copper-deficient rats

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Severely copper-deficient rats, derived from dams depleted during gestation and lactation, develop neurological signs associated with low dopamine in the corpus striatum. This investigation was designed to assess neuronal death by use of an astrocyte marker, glial fibrillary acidic protein (GFAP), and to determine possible correlation of liver and striatal copper with striatal dopamine. Virgin females were fed low copper diets containing sucrose or glucose (0.5 mg Cu/kg) or control diets (10 mg Cu/kg) during gestation and lactation. The offspring were fed the same diets until clinical signs appeared in the deficient groups, when they were 7–8 weeks of age. The corpus striatum was analyzed for dopamine and copper; striatum, cerebral cortex, and cerebellum were analyzed for GFAP. All rats fed the low copper diets had extremely low liver copper, <2 μg/g, dry, but only approximately one-half developed neuropathology and had low striatal dopamine. The GFAP concentration in the striatum increased in all copper-deficient rats, but was dramatically higher in those with low striatal dopamine. Cerebral GFAP was also higher in copper-deficient rats with low dopamine than in those with normal dopamine. There were no differences in cerebellar GFAP. Striatal copper correlated with striatal dopamine and was depressed to a greater extent in rats with high GFAP. The results suggest that the development of copper deficiency neuropathology is caused by neuronal degeneration that is associated with defective copper metabolism in the corpus striatum.

Keywords: Copper deficiency; neuropathology; striatal dopamine; striatal copper; glial fibrillary acidic protein; rat

Introduction

Neuropathology is a cardinal sign of severe copper deficiency induced by dietary copper deprivation of the dam during gestation and lactation, by deprivation of the weanling animal. Signs of central nervous system damage, including ataxia, akinesia, and tremors, have been observed in copper deficient sheep,[1] guinea pigs,[2] and rats.[3,4] Similar gross pathology occurs in genetic diseases, such as Menkes' disease in infants and the mottled mouse mutant, brindled, in which copper absorption and metabolism are impaired.[5,6] Decreased brain concentrations of norepinephrine have been observed in rats of low copper status due to dietary depletion[7–9] and in mice as a result of dietary deprivation[10] or genetic mutation.[10,11] Low brain dopamine also occurs in copper-deficient lambs[12] and rats[8,9,13] born to dams that consumed a low copper diet. These offspring exhibited gross neurological signs. Although decreased activity of the cuproenzyme dopamine-β-monooxygenase may explain the low concentration of norepinephrine and elevated concentration of dopamine in several tissues of copper-deficient animals,[14,15] the basis of the low concentration of dopamine in brain is unknown.

Some insight into the defective catecholamine metabolism is provided by the fact that the low norepinephrine concentration in the rat brain is readily

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restored to normal by copper repletion while the concentration of dopamine is not.[9] The results suggest that the activity of dopamine-β-monooxygenase is restored promptly by copper, but that the occurrence of structural or other nonreversible damage prevents restoration of dopamine levels. The corpus striatum is the richest source of dopamine in the brain, and the dopamine concentration in this region is most severely depressed by copper deficiency.[9] Similar gross and chemical pathology occurs in Parkinson's disease,[16] and is accompanied by massive loss of dopaminergic neurons.[17,18] The striatum serves as the terminus of dopaminergic neurons that arise in the substantia nigra of the midbrain. There tends to be a compensation for the dopamine deficit, perhaps by increasing receptor number and function, so that clinical signs in Parkinson patients and animal models do not appear until there is a loss of 70% or more of the neurons.[19]

In many pathological conditions of the central nervous system, including neuronal death, there is an increase in the concentration of astrocytes and of the intermediate-size filaments that are associated with astrocytes. These filaments are composed of glial fibrillary acidic protein (GFAP), a protein that serves as an astrocyte marker. Recently, Shafit-Zagardo et al.[20] observed a marked increase in the GFAP concentration in some brain regions of the brindled mouse, suggesting neuronal death or damage. Such damage could explain the neuropathology observed in copper-deficient rats.

The major objective of this study was to elucidate the mechanism by which copper deficiency in the rat causes neurological signs associated with decreased striatal dopamine concentration. The experimental design was based on the hypothesis that the neuropathology is caused by neuronal death and the subsequent proliferation of astrocytes. The concentration of GFAP was measured in brain parts of copper-adequate controls, and of neurologically susceptible and nonsusceptible copper-deficient rats from depleted dams (second-generation depleted). A secondary objective was to determine if the neurological signs observed in second-generation rats can be induced in undepleted weanling rats. Two sources of carbohydrate, sucrose and glucose, were compared because of the evidence that fructose and related sugars accentuate some signs of copper deficiency compared with glucose and starch.[21]

Materials and methods

Animals and diets

Albino rats of Wistar origin, produced in the departmental colony, which has been closed for 50 yrs, served as the source of all experimental animals. The major portion of the study involved rats whose dams were fed a low copper diet and are designated second-generation rats. They were produced by virgin females from the stock colony. The dams were fed the diets during gestation and lactation as described,[13] and the weanling offspring, male and female, were fed the same diets to an age of 7–8 wks. At this time offspring fed the low copper diets began to show neurological signs and their tissues, along with the respective controls, were collected for analysis. A corollary experiment utilized weanling rats (nutritionally first generation) obtained directly from the stock colony. These rats were fed the diets for a period of 11 weeks at which time mortality began to occur in the deficient groups. The experimental protocol was approved by the University of Missouri, Columbia Animal Care and Use Committee.

The experimental design was a 2 × 2 factorial with two sources of carbohydrate, sucrose and glucose, and two levels of added copper, 0 and 10 mg/kg. The diets were the same as previously described;[22] they contained 20% casein, 67.6% carbohydrate, 5% corn oil, and the essential vitamins and minerals. By analysis the basal diets contained approximately 0.5 mg copper/kg.

Tissue collection and sample preparation

The whole brain was quickly removed and kept on ice during dissection. The cerebellum was first removed and placed in a plastic vial kept on dry ice. This was followed by removal of corpus striatum and cerebral cortex. The striatum was normally analyzed for dopamine immediately, but on occasion was stored in liquid nitrogen for up to 3 days. Other tissue was stored at −20° C until analyzed.

GFAP analysis

For electrophoretic separation of cytosolic proteins and subsequent transblotting, brain tissue was first homogenized in glass with pH 8 buffer containing Tris, 50; EDTA, 2; phenylmethylsulfonyl fluoride (PMSF), 0.1 mmol/L. Inclusion of PMSF as a protease inhibitor was essential because preliminary experiments showed that without the inhibitor multiple immunoreactive bands resulted from endogenous proteolytic action. The homogenate was centrifuged for 45 min at 4° C and 100,000*g*. The resulting supernate, used for GFAP analysis, was analyzed for protein by the Lowry method,[23] using bovine serum albumin as the standard. Aliquots of the cytosolic protein (20 μg) were loaded in a 15-well, 12% polyacrylamide gel. The gels were prepared and electrophoresis performed according to Laemmli[24] using Protean II Slab Cell electrophoresis equipment (Bio-Rad, Richmond, CA USA). After separation of the protein bands, the proteins were transferred to nitrocellulose sheets using a Trans-Blot Cell and a constant voltage power supply (Bio-Rad).

For detection of GFAP on the nitrocellulose sheets, a specific immunological system similar to that of Shafit-Zagardo[20] was used. To block nonspecific binding sites before exposure to antibodies, the protein-loaded nitrocellulose sheets were soaked for 30 min. in a solution of phosphate buffered saline containing 5% casein and 0.05% Tween 20 (PBST). The sheets were then reacted with a 1:500 dilution of the primary antibody, rabbit anti-human GFAP antiserum (Dakopatts, Carpinteria, CA USA). After five washes with PBST, the sheets were treated with a 1:1000 dilution of a second antibody, goat anti-rabbit IgG coupled with horseradish peroxidase. The blots were then washed and dried with a hair dryer. GFAP was visualized by peroxidase-catalyzed oxidation of chloronaphthol to produce color at the GFAP site. For this purpose, 4 mL of freshly prepared 4-chloronaphthol (12 mg in 4 mL of methanol) were added to 20 mL of Tris buffer (50 mmol/L Tris.HCl; 20 mmol/L NaCl, pH 7.4). To this freshly prepared solution we added 25 μL of 30% hydrogen peroxide. The blotted sheet was agitated in this solution until the color reached visual maximum; after

which the color began to fade if left in the light. The reaction was stopped immediately after color development by addition of a large volume of distilled water. GFAP quantitation was accomplished by laser densitometric scanning of the stained blots. Standard curves were prepared by relating the area under the densitometric scans to graded levels of purified GFAP obtained from ICN Immunologicals (Lisle, IL USA).

Two assays were performed. In the first trial cytosolic protein from the striata of four rats fed the −CuSuc and four fed the +CuSuc diet, along with GFAP standards, were loaded on to a gel, separated, transblotted, and immunostained. A comparable gel loaded with protein from rats fed glucose was run concurrently. In the second trial cytosolic protein from the striata of 15 rats fed sucrose-based diets were loaded on to one gel; five controls with normal striatal dopamine (+CuNDA), five deficient rats with normal dopamine (−CuNDA), and five with low dopamine (−CuLDA). Protein aliquots from the cerebrum and cerebellum were loaded similarly. GFAP standards were run on separate gels along with each of the sample gels. While this design does not permit strict comparison with the GFAP standards, it provided direct comparison of the GFAP concentrations in specific tissues.

Copper and dopamine analyses

Liver copper was determined by dry ashing 0.5–1.0 g of tissue at 500° C for 24 hrs after drying the sample at 110° C overnight. The ash was treated with 2 mL of 25% nitric acid by heating slowly to dryness on a hot plate. It was then reashed for 24 hrs and the residue taken up in 0.1% nitric acid. The copper concentration in the solution was determined by use of a graphite atomic absorption spectrophotometer (Varian Zeeman SpectrAA, Mulgrave, Victoria, Australia). The copper concentration in small quantities of tissue, such as the striatum, was determined by the same method after wet ashing. Dried tissue was transferred to acid-washed pyrex tubes and 0.25 mL of sub-boiling nitric acid added. This acid was distilled from an all quartz still at a temperature below the boiling point. The sample was covered with a marble and pre-digested overnight at room temperature. A drop of 30% hydrogen peroxide was added and the tube heated in a block at 100° C for 4 hrs. The marble was then removed, the block temperature raised to 130°, and the solution evaporated to dryness. The samples were taken up in 0.1% nitric acid and copper determined as above. Values are reported on a dry weight basis. The procedure was certified by routine analysis of a standard bovine serum sample prepared in our laboratory.

Dopamine was determined by a slight modification of the method of Mefford,[25] using HPLC and electrochemical detection.[13] Quantitation was accomplished by use of a standard curve derived from graded levels (1.9–4.75 ng) of dopamine and an internal standard, 3,4-dihydroxybenzylamine hydrobromide (DHBA). The corpus striatum was homogenized in 0.5 mL of solution containing 1 mmol/L Na_2EDTA, 0.1 mmol/L perchloric acid, and 0.91 μmol/L of DHBA. The catecholamines were purified by adsorption onto alumina and elution with an EDTA-perchloric acid solution.

Statistical analysis

The data were analyzed by the analysis of variance (ANOVA) followed by a post-hoc *t* test using CRISP software (Crunch Software, San Francisco, CA USA).[26] Values of P less than 0.05 were considered statistically significant.

Results

Second-generation rats

The copper status of nutritionally second-generation rats studied in this experiment was evaluated by the concentration of liver copper and the hematocrit value obtained at the end of the trial. *Table 1* summarizes the results. Regardless of sex and the source of dietary carbohydrate, rats that consumed the low copper diets had extremely low concentrations of liver copper and low hematocrits. There was no copper × carbohydrate interaction as regards liver copper, but rats fed diets containing low copper and glucose had lower hematocrits than those fed similar diets with sucrose. This trend was statistically significant among females (copper × carbohydrate × sex, $P < 0.05$).

As in previous studies,[13,22] there was a wide distribution of striatal dopamine concentrations among rats fed low copper diets. The results are shown in *Figure 1*. The animals analyzed here were part of a larger study involving a total of 147 rats.[22] Analysis of the total population, as well as the animals presented in the figure, showed no sex effect as regards striatal dopamine concentration ($P > 0.8$) or incidence of clinical signs. Hence, data from males and females used in this study were pooled. Low striatal dopamine was highly correlated with the neurological signs observed before tissues were taken for analysis. Animals that exhibited definitive neurological signs and low dopamine accounted for approximately one-half of the

Table 1 Indices of copper status of rats fed the diets and derived from dams fed the same diets during gestation and lactation[a]

Dietary treatment	Liver copper[b]		Hematocrit[c]	
	Males	Females	Males	Females
+CuSuc	12.1 ± 1.5 (5)	10.2 ± 0.68 (5)	46 ± 2.3 (5)	43 ± 0.5 (3)
−CuSuc	1.24 ± 0.19 (12)	0.83 ± 0.09 (10)	18 ± 2.9 (3)	23 ± 4.2 (6)
+CuGlc	9.73 ± 0.21 (4)	13.9 ± 2.0 (5)	45 ± 1.1 (4)	47 ± 2.6 (5)
−CuGlc	1.07 ± 0.25 (4)	1.74 ± 0.54 (5)	16 ± 2.9 (8)	14 ± 3.0 (10)

[a]Hematocrits were determined and liver collected for analysis when definitive neurological signs appeared, 7–8 weeks of age.
[b]Means ± SEM, expressed as μg/g dry weight. ANOVA (three-way) showed a significant copper effect ($P < 0.01$), but no significant sex effect, no copper × carbohydrate interaction, and no copper × carbohydrate × sex effect. Number of observations shown in parenthesis.
[c]Means ± SEM, expressed as percentage of packed red cells. ANOVA showed a copper effect, a carbohydrate effect, and a copper × carbohydrate interaction ($P < 0.01$).

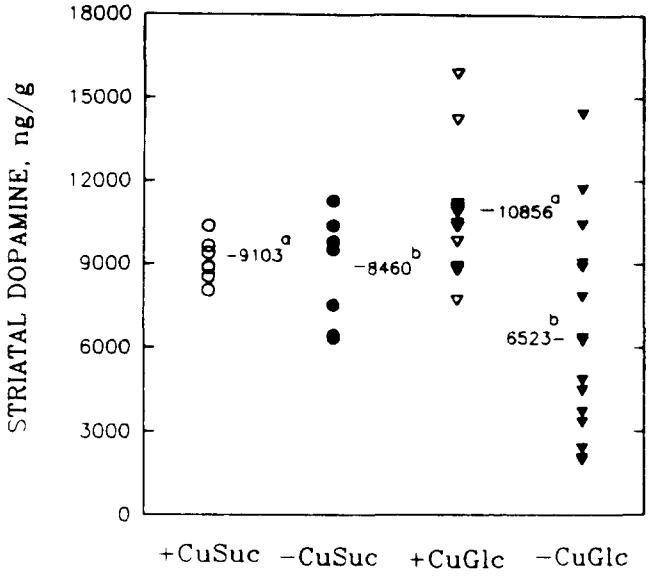

Figure 1 Distribution of dopamine concentrations in the corpus striatum of nutritionally second-generation rats of both sexes fed two sources of carbohydrate (sucrose and glucose) and low (−Cu) or adequate (+Cu) copper levels. Three-way ANOVA showed a significant copper effect ($P < 0.01$) but no carbohydrate effect, no sex effect, and no interactions. Means are presented for each treatment; different letter superscripts indicate statistical significance ($P < 0.05$).

population sampled. While the means were significantly different, a portion of the copper deficient (−Cu) rats had normal striatal dopamine concentrations and will be referred to here as copper deficient, normal dopamine (−CuNDA). Those copper deficient rats whose striatal dopamine were approximately two standard deviations or more below the control mean[13] will be designated as copper deficient, low dopamine (−CuLDA).

Glial fibrillary acidic protein was detected in cytosolic fraction of all brain parts tested, striatum, cerebrum, and cerebellum. It was not possible to make quantitative comparisons in trial 1, but qualitatively the striata of randomly selected rats from the −Cu groups contained appreciably more GFAP than those fed the +Cu diets. Photographs of the stained nitrocellulose sheets from trial 2 are shown in *Figure 2*. The measured densities of the bands from control rats were lowest in the striatum and highest in the cerebellum. These densities were compared to standards, photographs of which are shown at the bottom of the figure. The results are summarized in *Figure 3*. Copper deficiency significantly increased the concentration of GFAP in the striatum but had no effect on the cerebellum concentration. Perhaps most significant is the fact that the copper-deficient rats with low striatal dopamine (−CuLDA) had higher concentrations of GFAP in both striatum and cerebrum than deficient rats with normal dopamine levels (−CuNDA).

Liver copper concentration has been considered the best criterion of copper status, and a level significantly less than 2 μg/g, dry weight, is used here to define severe copper deficiency. As shown in *Figure 4*, there was no difference in the liver copper concentration between copper-deficient rats that had low striatal dopamine and those with normal dopamine concentration. However, the low dopamine group had significantly lower copper in the striatum than the normal dopamine group. Among the copper deficient rats there was a high correlation between striatal copper and dopamine concentrations. (See legend for Figure 4.)

First-generation rats

The results obtained with undepleted rats fed the experimental diets from weaning are presented in *Table 2*. Rats fed the low copper diets regardless of carbohydrate source grew slowly and had enlarged hearts, typical of copper deficiency. Except for females fed the −CuSuc diet, hematocrits were also low in rats deprived of copper. Mortality began to occur during week 12, at which time the experiment was terminated. In spite of the overt signs of copper deficiency, copper status had no effect on the striatal dopamine concen-

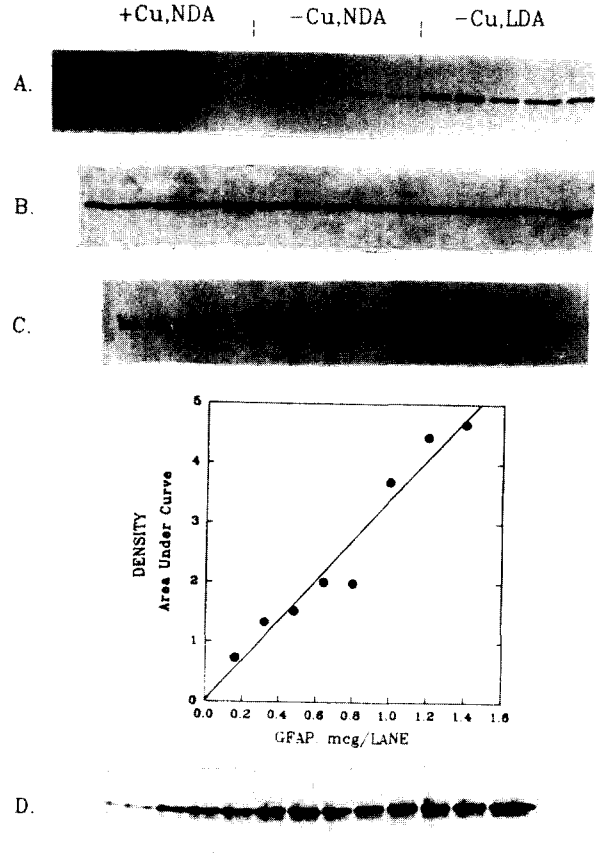

Figure 2 Photographs of glial fibrillary acidic protein (GFAP) immunoblots and a standard curve for quantitation of GFAP concentration. Shown are five samples from each of three groups, +CuNDA, −CuNDA, and −CuLDA, of both sexes. The brain areas sampled were corpus striatum (A), cerebral cortex (B), and cerebellum (C). A photograph of the standard GFAP blots (D) shows the concentration range from 0.16–1.2 μg per lane.

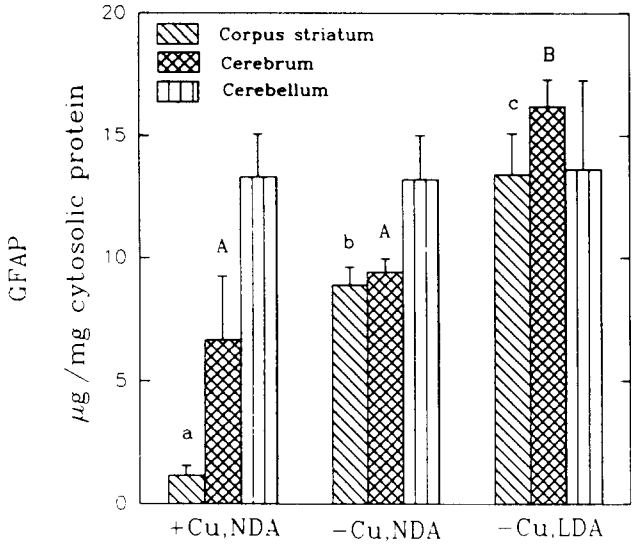

Figure 3 Concentrations of GFAP in the cytosolic fraction of corpus striatum, cerebral cortex, and cerebellum from +CuNDA, −CuNDA, and −CuLDA rats of both sexes (n = 5). The bars represent means, and the crossbar extensions represent the standard error of the mean. Different letters of the same case designate significant differences, $P < 0.05$.

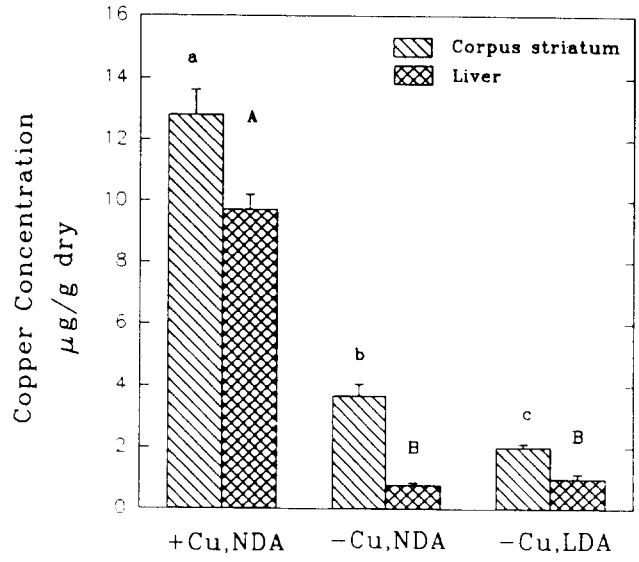

Figure 4 Copper concentrations in liver and corpus striatum of +CuNDA, −CuNDA, and −CuLDA rats of both sexes (n = 7–12). Designations as in *Figure 3*. There was a high correlation between the copper and dopamine concentrations in the striata of rats fed the low copper diets: DA = 2894 × Cu − 556; $r^2 = 0.866$, $P < 0.001$.

Table 2 Indices of copper status and striatal dopamine concentrations in rats fed the experimental diets from weaning[a]

Dietary treatment	Body weight[b]		Hematocrit[c]		Dopamine[d]	
	Males	Females	Males	Females	Males	Females
	g, 11 wks		% packed cells		ng/g	
+CuSuc	397 ± 3 (5)	244 ± 9 (3)	48 ± 0.4	46 ± 0.7	10100 ± 580	12300 ± 1180
−CuSuc	286 ± 20 (6)	221 ± 9 (3)	25 ± 6.1	39 ± 2.7	10400 ± 420	10500 ± 90
+CuGlc	389 ± 14 (3)	236 ± 9 (5)	45 ± 1.3	47 ± 0.9	17500 ± 710	13400 ± 720
−CuGlc	269 ± 15 (5)	209 ± 10 (7)	18 ± 2.1	28 ± 6.2	17900 ± 1730	14100 ± 540

[a]Means ± SEM; numbers in parenthesis designate animals analyzed at end of an 11-week experimental period. There were three deaths during the final week, two in the −CuSuc group and one in the −CuGlc group.
[b]ANOVA (three-way) showed a significant copper effect ($P < 0.01$), but no copper × carbohydrate effect.
[c]ANOVA showed a significant copper effect ($P < 0.01$), significant sex and carbohydrate effects ($P < 0.02$), but no copper × carbohydrate interaction.
[d]ANOVA showed a significant carbohydrate effect ($P < 0.01$), a significant sex effect ($P < 0.02$), but no copper effect.

tration. Dopamine concentration was significantly ($P < 0.01$) affected by carbohydrate source, but there was no copper × carbohydrate interaction.

Discussion

The low copper diets produced severe copper deficiency in the second-generation rats as shown by the indices summarized in *Table 1*. Liver copper and the hematocrit values were markedly depressed. The source of carbohydrate had no effect on the liver copper or severity of the neurological signs. Body weights were also depressed in rats fed the low copper diets. This was no doubt related to reduced food intake, which could conceivably induce neurological signs. However, in earlier experiments[13] it was shown that restricting the food consumption of control (+Cu) rats to that of copper-deficient pair-mates had no effect on striatal dopamine and did not induce neurological signs. The Parkinson-like signs and low dopamine levels observed here and earlier[8,9,13] are related to decreased copper intake and specifically to low striatal copper concentration.

The concentration of striatal GFAP was positively associated with the clinical pathology and low striatal dopamine concentration in the copper deficient rats. This is analogous to the markedly higher level of immunoreactive GFAP observed at postnatal day 12 in the brains of the male brindled mouse compared with controls.[20] In that study, Western blot analysis revealed major GFAP changes in the forebrain and cerebellum, the only areas studied. However, contrary to present

results, immunocytochemistry showed little GFAP change in the striatum or hypothalamus. It has been observed that the neurotoxin methylphenyltetrahydropyridine, which induces a Parkinson-like syndrome in humans and some animals, causes increased GFAP levels in the mouse striatum, accompanied by decreased dopamine concentration.[27] Our observations relating Parkinson-like signs and elevated GFAP in the striatum of copper deficient rats are similar to those of Reinhard et al.[27] GFAP is a recognized marker of astrocytes and an increase in its concentration in the brain suggests increased astrocyte number or metabolic activity. GFAP concentration in the brain increases as a result of mechanical injury or treatment with neurotoxicants, such as trimethyltin.[28,29] The increase is associated with elevated astrocyte number or metabolic activity and neuronal death. The increase in GFAP observed here is likely the result of neuronal death or damage.

The neuropathology and low striatal dopamine in our rats that were subjected to copper deficiency appear to have a genetic component, in as much as only approximately one-half of the offspring are affected. Susceptibility is litter related;[30] a dam that produces susceptible offspring in the first litter continues to do so, and one that produces nonsusceptible pups continues to do so for three or more litters.[30] Based on liver copper concentration, which is judged to be the most reliable index of copper status, all rats fed the low copper diet were severely copper deficient, regardless of whether they had low or normal striatal dopamine levels *(Figure 4)*. It is notable that in these rats there was a difference in the striatal copper concentration between those that had low striatal dopamine and those with normal dopamine. There was no difference in the liver copper concentration. This important observation confirms earlier results.[30] Striatal copper concentration was significantly lower in the copper-deficient rats that had low striatal dopamine. The results suggest that the genetically susceptible rats have a metabolic defect in the brain related to absorption of copper, or its binding and utilization. There was no difference in the copper concentration of the hypothalamus;[30] the effect in other areas of the brain is unknown.

Neurological disorders occur in severely copper-deficient rats, whether the deficiency is of genetic origin as in the brindled mouse or of nutritional origin as described here. It is pertinent to point out that in both cases the defect appears in offspring deprived during gestation. The results suggest that copper deficiency during fetal development is essential for the development of neuropathology. To test this hypothesis, we have produced severe copper deficiency in first-generation rats fed the same diets as the second-generation rats for 11 weeks. There were signs of severe copper deficiency, including mortality, among those fed the low copper diets, but there was no effect on striatal dopamine concentration. The mechanism by which in utero exposure to the low maternal copper status affects the central nervous system of the fetus should be explored. The results presented here support the concept that the genetically susceptible offspring, but not all second-generation, copper-deficient rats suffer loss of dopaminergic neurons when subjected to copper deprivation during gestation and lactation. Only copper-deprived rats with low striatal copper show clinical signs and have a low striatal dopamine concentration.

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