BUTTERWORTH HEINEMANN

Persistent neurochemical changes following perinatal copper deficiency in rats

Joseph R. Prohaska and William R. Bailey

Department of Biochemistry and Molecular Biology, University of Minnesota, Duluth, MN USA

Copper (Cu) levels in the central nervous system are known to be influenced by Cu nutriture during perinatal development. A rat model of dietary Cu deficiency (−Cu), initiated during gestation, was employed to examine changes in regional levels of brain Cu, norepinephrine (NE), dopamine (DA) and selected enzymes of month-old female Sprague Dawley offspring. Nutritional repletion of Cu to −Cu rats was studied following 1, 2, or 4 months. Levels of Cu in six different regions of brain from −Cu rats were reduced 80% or greater compared with levels in Cu-adequate (+Cu) controls. Following 4 months of Cu repletion, brain regional Cu levels were still below those measured in +Cu rats. The norepinephrine/dopamine was higher in +Cu compared with −Cu rats for all brain regions except corpus striatum suggesting impairment of dopamine-β-monooxygenase (DBM). Cu repletion normalized these ratios after 1 month. In vitro DBM activity was higher in samples from −Cu compared with +Cu rats and these differences were reversed by Cu supplementation. Cytochrome c oxidase (CCO) activity of 1-month-old −Cu rats was 18 to 50% of that measured in +Cu rats. Small differences in CCO activity remained in all regions, except hypothalamus, even after 4 months of repletion. Cu,Zn-superoxide dismutase activity (Cu,Zn-SOD) in −Cu rats was 75 to 91% of that measured in +Cu rats and was equivalent following repletion for 1 month. Four months of Cu repletion were not sufficient to restore brain Cu and CCO levels of 1-month-old −Cu rats. (J. Nutr. Biochem. 6:275–280, 1995.)

Keywords: copper deficiency; cuproenzymes; rat brain catecholamines; regional copper

Introduction

Copper (Cu) is one of several essential trace elements that are required during perinatal development of the central nervous system (CNS).[1] The essentialness of Cu for proper brain development of humans is best illustrated in children who have Menkes' disease, a genetic mutation that is characterized by severe neuronal degeneration and Cu deficiency.[2] The condition enzootic neonatal ataxia in sheep is another example that illustrates the essentialness of Cu during early brain development.[3] Laboratory-induced dietary Cu deficiency has been studied in guinea pigs and laboratory rodents and has pronounced affects on development and morphology of the brain. These studies are reviewed elsewhere.[4]

The abnormal development and morphology of the Cu-deficient brain may be the result of changes in specific cuproenzymes. There are a number of Cu-dependent proteins whose activity could change accompanying Cu deficiency. The consequences of these changes may alter the development and function of the brain. These cuproenzymes include: (1) Cu,Zn-superoxide dismutase (SOD) (EC 1.15.1.1); (2) cytochrome *c* oxidase (CCO) (EC 1.9.3.1); (3) dopamine-β-monooxygenase (DBM) (EC 1.14.17.1); (4) peptidylglycine-α-hydroxylating monooxygenase (PHM) (EC 1.14.17.3). These known cuproproteins only account for a portion of the total Cu in the CNS and thus other Cu-dependent functions undoubtedly exist. Which, if any, of these cuproproteins are most important in the etiology of the damaged CNS is presently unknown.

The impact of changes in the activity of some of the above-mentioned cuproenzymes have been studied in experimental Cu deficiency. Although reductions in the activity of SOD were noted in the brains of Cu-deficient rats,

Address reprint requests to Dr. J.R. Prohaska, Department of Biochemistry and Molecular Biology, University of Minnesota—Duluth, Duluth, MN 55812 USA.
Received July 15, 1994; revised October 14, 1994; accepted November 27, 1994.

Nutritional Biochemistry 6:275–280, 1995

655 Avenue of the Americas, New York, NY 10010

0955-2863/95/$10.00
SSDI 0955-2863(95)00036-Y

there was no evidence for limitation of this important antioxidant enzyme as assessed by measuring lipid peroxidation.[5,6] Similarly, although there were major reductions in the activity of brain CCO in Cu-deficient rat pups, there was no limitation in the steady-state levels of ATP, the ultimate product of the CCO reaction.[5,6] PHM activity was not lowered by dietary Cu deficiency in the young adult rat model studied.[7]

More extensive studies have been conducted on the cuproprotein DBM. Measurement of DBM activity from brains of Cu-deficient mice[8] and rats[9] indicates higher activity compared with control samples. However, high performance liquid chromatography (HPLC) analysis of brain tissue from both mice and rats indicates that DBM activity is actually lower in the brains of Cu-deficient animals. This is indicated by a decrease in the concentration of norepinephrine, the product of the reaction, and an increase in dopamine, the substrate of DBM. Some have reported that brain dopamine is lower in the brains of Cu-deficient rats.[10,11] These changes in brain catecholamines in Cu-deficient mice and rats appear to be regionally specific.[9,12] The regional specificity is an important consideration given the heterogeneity and the distribution of both noradrenergic and dopaminergic neurons in the CNS.

There have been a limited number of repletion experiments in which Cu-deficient mice and rats have been nutritionally supplemented for short periods of time to investigate repletion of cuproenzyme activities. A 1 month supplementation period to 1-month-old Cu-deficient rats did not restore brain Cu nor CCO activity, whereas norepinephrine levels were elevated to control values.[6,13] Similar results have been recently obtained following 1-month repletion of Cu-deficient mice.[12,14] In the mouse studies, some regional measurements were made. They indicated that SOD and catecholamine levels were returned to normal within 1 month whereas CCO activity and Cu levels were not.

We have recently completed a study of nutritional Cu deficiency during perinatal development in rats in which major reductions in regional Cu and alterations in catecholamines where documented.[9] It is the purpose of the current studies to extend this nutritional paradigm by following repletion with Cu for a long period of time and to monitor not only the concentration of Cu in the brain regions but also that of specific cuproenzymes, namely SOD, CCO, and DBM.

Methods and materials

Animals and diets

Pregnant Sprague–Dawley rats were purchased commercially (Harlan Sprague–Dawley, Indianapolis, IN USA), and 2 days following parturition liter size was adjusted to 8 pups.

Dietary treatments began 7 days into gestation and pups were weaned at 20 days and transferred to stainless steel cages and housed individually on the same treatment as their respective dams for an additional 9 days. One-month-old male and female offspring from 8 litters of Cu-deficient and 6 litters of Cu-adequate control dams were randomly sampled to establish Cu status. This protocol is similar to that recently used to study neurochemical changes following perinatal Cu deficiency.[9] Remaining female offspring from the two dietary groups were offered a nonpurified commercial diet, Purina LRC 5001, and tap water for 4 months. Insufficient numbers of male offspring prevented their inclusion in the repletion study. Cu-adequate and Cu-repleted female rats were sampled at age 2, 3, and 5 months.

Rat dams and young offspring were maintained on one of two dietary treatments, Cu-deficient or Cu-adequate, that consisted of feeding a Cu-deficient purified diet (Teklad Laboratories, Madison, WI USA) and either low Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet was formulated according to the AIN-76A diet and contained the following major components (g/kg of diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; AIN-76A vitamin mix, 10; DL-methionine, 3; choline bitartrate, 2; and ethoxyquin, 0.01.[15,16] Cupric carbonate was omitted from the AIN-76 mineral mix. Offspring and dams on the Cu-deficient treatment drank deionized water whereas Cu-adequate treatment groups drank water that contained 20 mg of Cu/L by adding Cu to the drinking water as $CuSO_4$. Diet and drinking water were available ad libitum. The modified AIN-76A diet contained 0.34 mg of Cu/kg whereas the LRC 5001 contained 13.6 mg of Cu/kg by chemical analysis.

Rats were maintained at 24°C with 55% relative humidity on a 12-hr light cycle (0700 to 1900 hr) in an AAALAC accredited facility. All protocols were formally approved by the University of Minnesota Animal Care Committee.

Sample collection

Rats were sampled at ages 1, 2, 3, and 5 months. Blood samples were drawn into heparinized microhematocrit tubes from trunk blood following decapitation after light ether anesthesia. A small aliquot was also removed for hemoglobin analysis. Plasma was obtained by centrifugation. Brains were bisected saggitally and dissected on a chilled glass plate into six regions following the guidelines of Glowinski and Iversen (1966).[17] The six regions were cerebellum, medulla oblongata + pons, cerebrum (cortex), hypothalamus, corpus striatum, and midbrain + hippocampus. Livers were removed, rinsed with deionized water, weighed, and a portion was processed for metal analysis. For enzymatic analysis, brain regions were homogenized for 30 sec in 9 or 24 vol of 0.05 mol/L of potassium phosphate (pH 7.0) with a Tissumizer and microprobe (SDT-080EN, Tekmar Co., Cincinnati, OH USA).

Chemical analyses

Portions of liver and 1-g samples of diets were wet-digested with 4 mL of concentrated HNO_3 (AR select grade, Mallinckrodt, St. Louis, MO USA), and the residue was brought to 4.0 mL with 0.1 mol/L of HNO_3. Samples were then analyzed for Cu and Fe by flame atomic absorption spectroscopy (Model 2380, Perkin-Elmer, Norwalk, CT USA). Brain regions were wet-digested in 5 mL micro Fernbach flasks by adding 1 mL of 3 mol/L of HNO_3 (Optima grade, Fisher Scientific, Pittsburgh, PA USA). The residue was suspended in 0.01 mol/L of HNO_3 and 15 µl aliquots of various dilutions were analyzed in duplicate by graphite furnace atomic absorption spectroscopy (HGA 400 AS-40, Perkin-Elmer) using method of additions. Cortex samples were analyzed by flame AAS.

Brain regional norepinephrine and dopamine levels were determined by reverse phase HPLC with ion pairing and electrochemical detection as described previously.[18] The molar ratios of norepinephrine to dopamine were calculated to better reflect the

activity of dopamine-β-monooxygenase.[9] Hemoglobin was determined spectrophotometrically as metcyanhemoglobin.[19] The total protein content was determined by analysis of the brain homogenates using a modified Lowry method with bovine albumin as a reference.[20]

Enzymatic analyses

Details of the enzyme assays are described elsewhere. Plasma was obtained from microhematocrit tubes following centrifugation and the activity of the cuproprotein ceruloplasmin (EC 1.16.3.1) was determined by measuring the ability of plasma to oxidize *o*-dianisidine using a modification, 37°C, of the method of Lehman et al.[21] DBM in vitro activity was determined spectrophotometrically on midbrain and medulla homogenates as described previously for mouse brain by measuring conversion of tyramine to octopamine.[8] SOD activity was measured by following inhibition of pyrogallol auto-oxidation at 320 nm as described previously.[19] Homogenates were treated with 0.4 vol of chloroform:ethanol (15:25) to inactivate manganese SOD. Cytochrome *c* oxidase activity was determined on fresh homogenates since the loss of activity upon storage was noted previously.[8] Homogenates were treated with 0.1% Triton X-100. Initial velocity was measured at 25°C and the rate of ferricytochrome *c* formation (μmol/min) was determined using a molar extinction coefficient of 19,600 for reduced-oxidized cytochrome *c*. Kinetic enzyme assays were run in duplicate with a temperature-controlled spectrophotometer (Beckman DU-8). Activities, with the exception of ceruloplasmin, were all expressed as U/mg of protein.

Statistical analysis

At each age, treatment means were compared by Student's *t*-test $\alpha = 0.05$ and $\alpha = 0.01$, using a Macintosh computer and statistical software (Statview 4.0, Abacus Concepts, Inc., Berkeley, CA USA). The statistical evaluation of age effects was not explored.

Results

Female offspring that were defined as being Cu-deficient were compared with control animals at 1 month of age (*Table 1*). These offspring exhibited characteristics of Cu deficiency such as loss of ceruloplasmin activity in the plasma and 10 fold reduction in the liver Cu concentration. In this particular set of animals there was no difference in body weight and the females were not anemic. The features of these 1-month-old female animals are similar to those described in the previous work with this experimental model.[9] Females were sampled after 1, 2, or 4 months of repletion on a nutritionally adequate, commercial diet (*Table 1*). Of the 35 Cu-deficient females obtained from 8 litters, 10 died during the first 3 days of repletion. No mortality was observed in 19 Cu-adequate females obtained from 6 litters. It is apparent that as little as 1 month of repletion restored the deficit in ceruloplasmin activity and liver Cu back to control levels in the survivors. In general the female animals were similar throughout the sampling period for the biochemical analyses exclusive of the CNS.

Brains from samples of the populations of female rats were dissected into six specific regions and analyzed for total Cu by graphite furnace atomic absorption spectroscopy. One-month-old Cu-deficient offspring had severely depleted brain Cu levels in all six regions, and the restoration of the Cu pools slowly rose with age but did not reach control levels, even after 4 months of repletion (*Figure 1*). The Cu concentration in the six regions from Cu-repleted rats averaged 67 ± 7% of those from Cu-adequate controls. The pattern of repletion in the six different brain regions was not remarkably different. In the control animals there was a slow rise in the concentration of Cu with age between 1 and 5 months although this rise in the concentration of Cu was not particularly striking with the exception of the hypothalamus (*Figure 1*).

To determine whether or not the restoration of brain Cu was still affecting functional Cu pools, the activity of three cuproenzymes was determined. Protein concentration was not altered by dietary Cu deficiency, and values were similar at all times tested between the two treatment groups (data not shown). The specific activity of CCO in the six regions studied was significantly lower in the 1-month-old female Cu-deficient rats compared with controls and seemed to be least affected in the hypothalamus (*Figure 2*). Repletion of Cu in the diet of these animals resulted in a rise in CCO activity at age 3 and 5 months. However, only in the hypothalamus was full restoration of enzyme activity

Table 1 Features of female rats in repletion study

Parameter	Group	Age (months) 1	2	3	5
Body weight (g)	CuA	95.5 ± 4.8	210 ± 21.6	242 ± 10.6	267 ± 14.1
	CuR	82.5 ± 11.4	183 ± 14.4	237 ± 15.7	288 ± 16.4
Brain/body (mg/g)	CuA	16.2 ± 0.71	8.17 ± 0.68	7.59 ± 0.35	7.12 ± 0.49
	CuR	18.2 ± 2.09	8.88 ± 0.65	7.29 ± 0.50	6.37 ± 0.22
Hemoglobin (g/100 mL)	CuA	11.0 ± 0.73	13.8 ± 1.1	13.0 ± 0.7	13.4 ± 0.3
	CuR	10.9 ± 0.63	13.1 ± 0.5	12.7 ± 0.5	13.3 ± 0.7
Ceruloplasmin (U/L)	CuA	77.6 ± 22.4	176 ± 22.2	228 ± 19.5	249 ± 21.7
	CuR	0.04 ± 0.07*	167 ± 8.7	234 ± 14.1	231 ± 29.4
Liver Cu (μg/g of fresh weight)	CuA	8.78 ± 3.28	5.00 ± 1.4	4.11 ± 0.27	4.04 ± 0.17
	CuR	0.72 ± 0.15*	4.29 ± 0.66	4.01 ± 0.20	4.00 ± 0.10

Values are means ± SD (n = 3–5). Means were compared by Student's *t*-test *$P < 0.01$. One-month-old female rats were offered Purina LRC 5001 and tap water for 4 months. Control Cu-adequate (CuA) rats were compared with Cu-deficient rats that began repletion (CuR) at 30 days of age. Details of the dietary treatments prior to repletion are listed in the Methods.

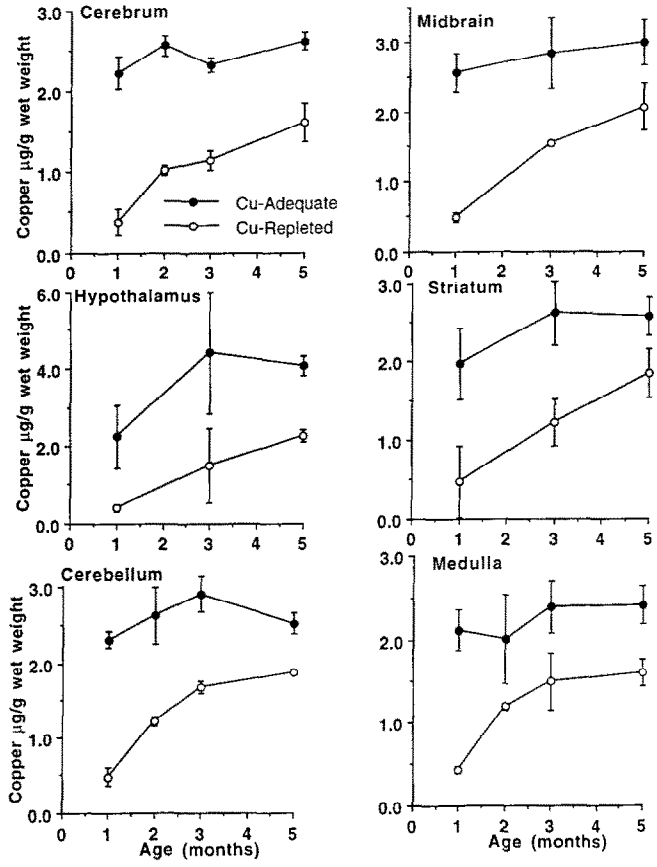

Figure 1 Brain regional levels of Cu in female Sprague–Dawley rats (n = 3–5). One-month-old Cu-deficient rats were offered a Cu-adequate diet (Purina LRC 5001) and compared with Cu-adequate controls fed the same diet. Points are means ± SD. Values of Cu-deficient (1 month) and Cu-repleted rats were lower than Cu-adequate at all times tested; $P < 0.05$.

accomplished in the 4-month recovery period (*Figure 2*). The second cuproenzyme, SOD, was also examined in these experiments. SOD activity was significantly lower in brains from Cu-deficient rats than controls (*Figure 3*). However, the absolute difference between the groups was very small (in all regions, except the cerebellum, less than 15% reduced), and the enzyme activity was rapidly restored upon feeding a diet adequate in Cu (*Figure 3*). No differences existed at 3 or 5 months of age between the control and the Cu-repleted groups with the single exception of the striatum at 5 months of age. The third cuproenzyme that was investigated in these studies was DBM. Consistent with previous results, the enzymatic activity of DBM in both the midbrain and medulla pons was significantly elevated, 32% and 76% respectively, in the Cu-deficient, 1-month-old female offspring (*Table 2*). Only these two regions were studied in these experiments. Upon Cu supplementation, this elevation in activity disappeared and there were no differences between the two groups at age 3 or 5 months (*Table 2*). However, when regional analysis of brain catecholamines was performed, a limitation in DBM activity was observed in the samples from Cu-deficient rats (*Figure 4*). In five of the six regions examined there was a significant elevation in the ratio of norepinephrine to dopamine in the Cu-adequate samples. This was most evident in the cerebellum and medulla-pons (*Figure 4*). These differences in catecholamine ratios disappeared rapidly upon repletion with dietary Cu in all regions studied. The one brain region that did not show a significant difference in the ratio of norepinephrine to dopamine was the corpus striatum, a region rich in dopamine and low in DBM activity (data not shown). For the most part the ratio of norepinephrine to dopamine was similar in all six brain regions studied over the period of 1 month to 5 months of age in control rats.

Discussion

The most striking result of these studies with female Sprague–Dawley rats is the very slow recovery of brain Cu following repletion of perinatal Cu-deficiency. Four months of nutritional repletion with an excess of Cu was not sufficient to restore the brain pool size back to normal, although plasma and liver pools were likely restored within days. These studies thus extend the earlier observations in both rats and mice that were conducted for a 1-month restoration period.[6,12–14]

The depletion of brain Cu in the 1-month-old animals

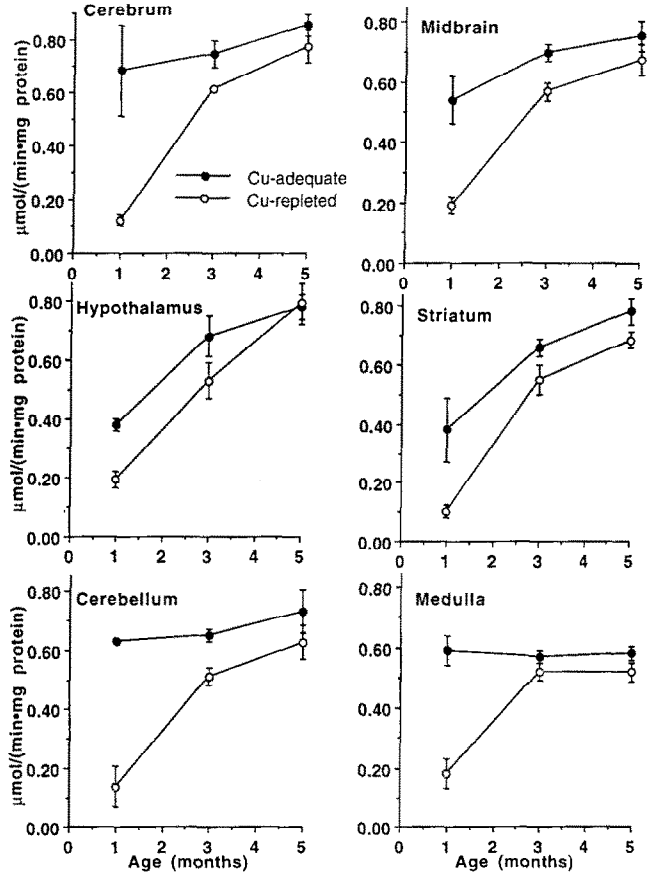

Figure 2 Brain regional levels of cytochrome c oxidase activity in female Sprague–Dawley rats (n = 3–5). One-month-old Cu-deficient rats were offered a Cu-adequate diet (Purina LRC 5001) and compared with Cu-adequate controls fed the same diet. Points are means ± SD. Values of Cu-deficient (1 month) and Cu-repleted rats were lower than Cu-adequate at all times tested, except the hypothalamus at 5 months of age; $P < 0.05$.

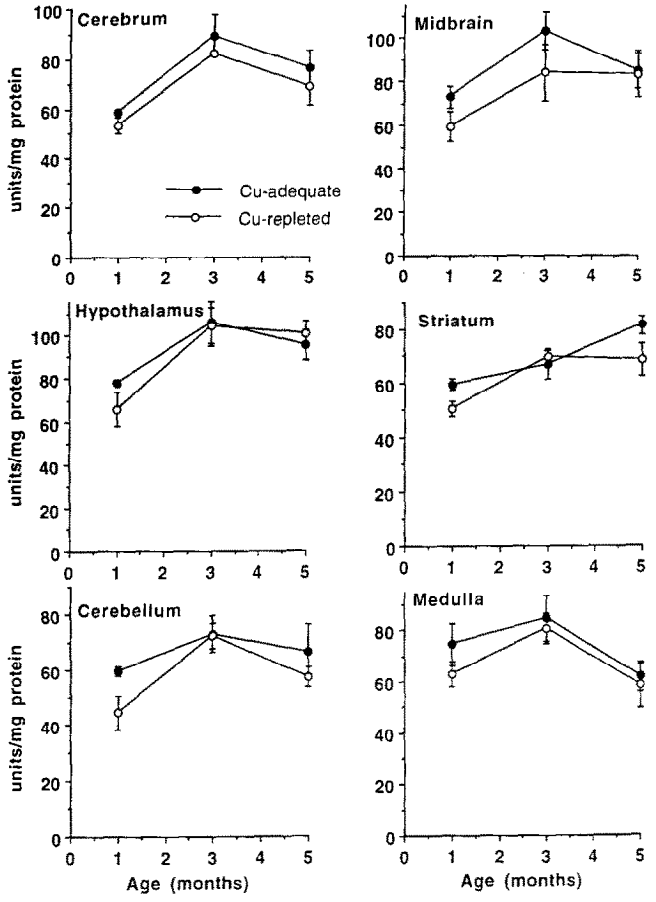

Figure 3 Brain regional levels of Cu,Zn-superoxide dismutate activity in female Sprague–Dawley rats (n = 3–5). One-month-old Cu-deficient rats were offered a Cu-adequate diet (Purina LRC 5001) and compared with Cu-adequate controls fed the same diet. Points are means ± SD. Values of Cu-deficient (1 month) and Cu-repleted rats for striatum at 5 months of age were lower than Cu-adequate means; $P < 0.05$.

was rather uniform in the six regions examined. This is similar to what was observed in mice[12] but somewhat different than what was previously observed in rats.[9] In the previous studies with rats it appeared that the hypothalamus had somewhat more modest reductions in brain Cu in both male and female offspring. This was not clear in the sample size in the current studies. The levels of depletion in the other five regions were similar to that observed previously.[9] Recovery of the Cu pools in all six regions studied seemed to be approximately equivalent in the current experiments. It is not known why such a slow recovery takes place. It is possible that the Cu deficiency during early development altered the blood-brain-barrier transport system, thus there is an impairment in uptake of Cu from plasma into the brain. It is also known that the turnover of Cu in the brain is extremely slow and thus it may take longer than 4 months to restore the pool size to control levels.[22] It is not known what the consequences of this lack of Cu in the brain might be.

Certainly there are persistent neurochemical changes in the brains of these repleted rats consistent with the limitation in some Cu-dependent function. This is evident in the case of the restoration of CCO activity, which was still below normal in five of the six brain regions even after 4 months of dietary Cu repletion. This is similar to earlier studies of 1-month repletion in which the CCO activity did

Table 2 Brain dopamine-β-monooxygenase activity of female rats in repletion study

Brain region	Group	Age (months) 1	3	5
Midbrain	CuA	0.71 ± 0.10	0.59 ± 0.07	1.15 ± 0.13
(nmol/h/mg)	CuR	0.94 ± 0.09*	0.64 ± 0.06	1.15 ± 0.05
Medulla-pons	CuA	1.14 ± 0.30	0.76 ± 0.11	1.65 ± 0.16
(nmol/h/mg)	CuR	2.01 ± 0.50*	0.81 ± 0.07	1.90 ± 0.25

Values are means ± SD (n = 4). Means were compared by Student's t-test *$P < 0.01$. Activity was determined spectrophotometrically as octopamine formation from tyramine. One-month-old female rats were offered Purina LRC 5001 and tap water for 4 months. Control Cu-adequate (CuA) rats were compared with Cu-deficient rats that began repletion (CuR) at 30 days of age. Details of the dietary treatments prior to repletion are listed in the Methods.

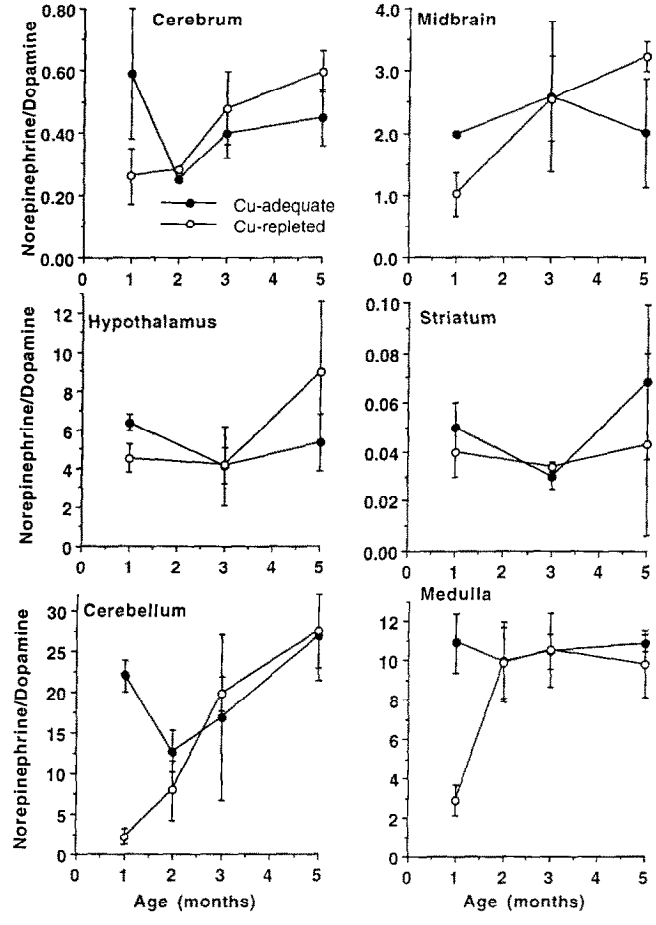

Figure 4 Brain regional molar ratios of catecholamines in female Sprague–Dawley rats (n = 3–5). One-month-old Cu-deficient rats were offered a Cu-adequate diet (Purina LRC 5001) and compared with Cu-adequate controls fed the same diet. Points are means ± SD. Values from Cu-deficient (1 month) rats, except for striatum, were lower than Cu-adequate means; $P < 0.05$.

not come back to normal in rat brain.[6] Also, earlier studies with mice indicated that CCO activity was still altered after 1 month of repletion, whereas SOD activity and catecholamine changes were totally reversed.[12] These observations in mice are consistent with the current studies in female rats. SOD activity in the brains of Cu-deficient rats was less affected than CCO activity and was rapidly restored following Cu repletion by diet. Measured changes in DBM activity and altered catecholamine ratios are consistent with recent work in rats.[9] The elevation of DBM activity was rapidly lowered to control levels by diet, suggesting that the alteration in enzyme activity was the result of dietary Cu deficiency. The rapid restoration of norepinephrine levels is also consistent with earlier work in rats[6,13] and mice.[12,14] It has not yet been established if the enzymatic activity of peptidyglycine α-hydroxlating monooxygenase is affected by perinatal Cu deficiency. There may be other Cu-dependent enzymes in brain whose activity is altered and susceptible to repletion.

These studies, which are extensions of earlier work on Cu deficiency in perinatal development, suggest the possibility that there are long-term consequences of Cu deficiency during fetal and lactational development. The potential severity would depend on the duration and degree of Cu deficiency. These developmental consequences may result in abnormal behavioral changes or physiological changes in the "recovered" animals. The testing of this hypothesis is the subject of current investigations. It is possible that these observations in experimental rodents have bearing on the human situation. Most of the fetal Cu in humans is transferred in the last trimester of pregnancy. Danks has commented that Cu deficiency in humans might occur if this fetal transfer period were compromised.[23]

Acknowledgments

Support for this research was provided by grants 91-37200-6319 and 93-37200-8756 from NRI Competitive Grants Program/USDA.

References

1 Prohaska, J.R. (1987). Functions of trace elements in brain metabolism. *Physiol. Rev.* **67,** 858–901

2 Danks, D.M., Campbell, P.E., Walker-Smith, J., Stevens, B.J., Gillespie, J.M., Bloomfield, J., and Turner, B. (1972). Menkes' kinky-hair syndrome. *Lancet* **1,** 1100–1103

3 Smith, R.M. (1983). Copper in the developing brain. In: *Neurobiology of the Trace Elements* (I.E. Dreosti, and R.M. Smith, eds.), p. 1–40, Humana Press, Clifton, NJ USA

4 O'Dell, B.L. and Prohaska, J.R. (1983). Biochemical aspects of copper deficiency in the nervous system. In: *Neurobiology of the Trace Elements* (I.E. Dreosti, and R.M. Smith, eds.), p. 41–81, Humana Press, Clifton, NJ USA

5 Prohaska, J.R. and Wells, W.W. (1974). Copper deficiency in the developing rat brain: A possible model for Menkes' steely-hair disease. *J. Neurochem.* **23,** 91–98

6 Prohaska, J.R. and Wells, W.W. (1975). Copper deficiency in the developing rat brain: evidence for abnormal mitochondria. *J. Neurochem.* **25,** 221–228

7 Mains, R.E., Myers, A.C., and Eipper, B.A. (1985). Hormonal, drug, and dietary factors affecting peptidyl glycine α-amidating monooxygenase activity in various tissues of the adult male rat. *Endocrinology* **116,** 2505–2515

8 Prohaska, J.R. and Smith, T.L. (1982). Effect of dietary or genetic copper deficiency on brain catecholamines, trace metals and enzymes in mice and rats. *J. Nutr.* **112,** 1706–1717

9 Prohaska, J.R. and Bailey, W.R. (1994). Regional specificity in alterations of rat brain copper and catecholamines following perinatal copper deficiency. *J. Neurochem.* **63,** 1551–1557

10 O'Dell, B.L., Smith, R.M., and King, R.A. (1976). Effect of copper status on brain neurotransmitter metabolism in the lamb. *J. Neurochem.* **26,** 451–455

11 Morgan, R.F. and O'Dell, B.L. (1977). Effect of copper deficiency on the concentrations of catecholamines and related enzyme activities in the rat brain. *J. Neurochem.* **28,** 207–213

12 Prohaska, J.R. and Bailey, W.R. (1993). Persistent regional changes in brain copper, cuproenzymes and catecholamines following perinatal copper deficiency in mice. *J. Nutr.* **123,** 1226–1234

13 Feller, D.J. and O'Dell, B.L. (1980). Dopamine and norepinephrine in discrete areas of the copper-deficient rat brain. *J. Neurochem.* **34,** 1259–1263

14 Prohaska, J.R. and Bailey, W.R. (1993). Copper deficiency during neonatal development alters mouse brain catecholamine levels. *Nutr. Res.* **13,** 331–338

15 American Institute of Nutrition (1977). Report of the AIN Ad Hoc Committee on standards for nutritional studies. *J. Nutr.* **107,** 1340–1348

16 American Institute of Nutrition (1980). Second report of the AIN Ad Hoc Committee on standards for nutritional studies. *J. Nutr.* **110,** 1726

17 Glowinski, J. and Iversen, L.L. (1966). Regional studies of catecholamines in the rat brain. *J. Neurochem.* **13,** 655–669

18 Prohaska, J.R., Bailey, W.R., Gross, A.M., and Korte, J.J. (1990). Effect of dietary copper deficiency on the distribution of dopamine and norepinephrine in mice and rats. *J. Nutr. Biochem.* **1,** 149–154

19 Prohaska, J.R. (1983). Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. *J. Nutr.* **113,** 2048–2058

20 Markwell, M.A.K., Haas, S.M., Bieber, L.L., and Tolbert, N.E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87,** 206–210

21 Lehman, H.P., Schosinsky, K.H., and Beeler, M.F. (1974). Standardization of serum ceruloplasmin concentrations in international units with *o*-dianisidine dihydrochloride as substrate. *Clin. Chem.* **20,** 1564–1567

22 Levenson, C.W., and Janghorbani, M. (1994). Long-term measurement of organ copper turnover in rats by continuous feeding of a stable isotope. *Anal. Biochem.* **221,** 243–249

23 Danks, D.M. (1988). Copper deficiency in humans. *Ann. Rev. Nutr.* **8,** 235–257