Alterations of Rat Brain Peptidylglycine α-Amidating Monooxygenase and Other Cuproenzyme Activities Following Perinatal Copper Deficiency (43929)

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Abstract. **Perinatal copper (Cu) deficiency was studied in month-old female and male Sprague-Dawley rat offspring to investigate regional changes in brain cuproenzymes. Offspring of dams given the low Cu treatment beginning at Day 7 of gestation exhibited signs characteristic of Cu deficiency including a 70% reduction in liver Cu levels compared with Cu-adequate controls. Compared with Cu-adequate rats, Cu-deficient rats had lower activities of the cuproenzymes peptidylglycine α-amidating monooxygenase (PAM), cytochrome c oxydase (CCO), and Cu,Zn-superoxide dismutase (SOD) in all six brain regions studied. Apparent activity of dopamine-β-monooxygenase (DBM) was higher in all regions from Cu-deficient compared with Cu-adequate rats. Activity of the selenoenzyme glutathione peroxidase (GPX) was not greatly altered in brain by Cu deficiency. Following 1 month of Cu repletion, liver but not brain Cu levels were equivalent to control. Brain CCO activity was still lower in Cu-repleted female and male rats. PAM activity was still lower in cerebrum of Cu-repleted rats. DBM activity was no longer significantly elevated in the former Cu-deficient groups except for midbrain. SOD and GPX activity were equivalent between groups. PAM activity, *in vitro,* is lower in the brain following perinatal Cu deficiency and activity is slow to recover following nutritional supplementation with Cu. Perhaps neuropeptide maturation is compromised by Cu deficiency.** [P.S.E.B.M. 1995, Vol 210]

Copper (Cu) is one of several essential metals required during perinatal development of the central nervous system (CNS) (1). In humans, the requirement of Cu for the brain is best exhibited by Menkes' disease, a rare neurological disorder first associated with Cu deficiency in 1972 (2). Severe nutritional Cu deficiency in humans is rare but may be a potential problem in premature infants especially in those with secondary absorption complications (3).

The neurochemical functions of Cu remain elusive. It is tempting to speculate that those functions are associated with known cuproenzymes. These enzymes include cytochrome c oxidase (CCO), dopamine-β-monooxygenase (DBM), and Cu,Zn-superoxide dismutase (SOD). Cu deficiency, studied in rodent models, is associated with alterations in the activity of all three of these enzymes (4). Can the neurological abnormalities accompanying Cu deficiency be explained by alterations in CCO, DBM, SOD, or other cuproenzymes (ceruloplasmin, lysyl oxidase, tyrosinase)?

Another potential candidate enzyme which might explain the diverse expression of Cu deficiency in the CNS and other biological systems is peptidylglycine α-amidating monooxygenase (PAM) (EC 1.14.17.3). PAM, like DBM, is a monooxygenase dependent on both ascorbate and Cu for activity (5). There is even sequence homology between PAM and DBM near the catalytic domain (6). PAM is responsible for the post-

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translational modification of a number of precursor neuropeptides which contain glycine at the COOH-terminus. PAM converts these precursors into the corresponding α-amide with release of glycine carbons as glyoxalate (7). Some of the more notable α-amidated peptides include gastrin, cholecystokinin, oxytocin, vasopressin, neuropeptide Y, vasoactive intestinal peptide, Substance P, and calcitonin. Since glycine-extended forms of the precursor of some of these peptides are known, Eipper *et al.* (7) suggest that levels of PAM can clearly be rate limiting under physiological conditions.

PAM is a bifunctional protein which catalyzes a two-step conversion of the glycine precursor. One domain, peptidylglycine α-hydroxylating monooxygenase (PHM), requires Cu for activity. The other domain, peptidyl-α-hydroxyglycine α-amidating lyase (PAL) (EC 4.3.2.5), does not. Some evidence suggests that alterations in Cu might influence PAM activity. Studies employing the Cu chelator diethyldithiocarbamate (DDC) have shown that PAM activity can be reduced *in vivo*. These studies demonstrated altered physiologic function, namely, gastrin and acid secretion (8), and Substance P and pain threshold (9). A single study on the effect of dietary Cu deficiency and PAM activity failed to find altered α-MSH levels (a PAM product) but did detect elevated PAM activity in some but not all tissues *in vitro* (10). This chronic Cu deficiency study, however, was initiated with adult rats, thus it is possible that changes to the CNS were minimal.

The objective of the current studies was to extend our perinatal Cu-deficient model (11) by examining brain regional PAM activity and comparing it with other cuproenzymes. A second objective was to determine if dietary Cu repletion could reverse putative neurochemical changes.

Methods and Materials

Animal Care and Diets. Sperm-positive rats, purchased commercially (Harlan Sprague-Dawley, Indianapolis, IN) received one of two dietary treatments, copper deficient or copper adequate, consisting of a Cu-deficient purified diet (Teklad Laboratories, Madison, WI) and either low Cu drinking water or Cu-supplemented drinking water, respectively. The purified diet was similar to the AIN-76A diet (12, 13) and contained the following major components (g/kg diet): sucrose, 500; casein, 200; cornstarch, 150; corn oil, 50; cellulose, 50; modified AIN-76 mineral mix, 35; and AIN-76A vitamin mix, 10. Cupric carbonate was omitted from the AIN-76 mineral mix. The purified diet contained 0.46 mg Cu/kg and 46 mg Fe/kg by chemical analysis. This diet contains approximately one-tenth of the optimal dietary Cu level for rats. Offspring and dams on the Cu-deficient treatment drank deionized water, whereas Cu-adequate treatment groups drank water that contained 20 μg Cu/ml by adding $CuSO_4$ to the drinking water. Rats were given free access to diet and drinking water. All animals were maintained at 24°C with 55% relative humidity on a 12:12-hr light:dark cycle (0700–1900 hr). All protocols were formally approved by the University of Minnesota Animal Care Committee.

Pregnant dams were placed on the Cu-deficient treatment 7 days after they were identified sperm positive. Two days following parturition litter size was adjusted to eight pups. Offspring were weaned when 3 weeks old, placed in stainless steel cages, and maintained on the same treatment as their respective dams for an additional 9 days. A total of 10 litters (five Cu adequate and five Cu deficient) were studied. This paradigm is similar to that recently used to study neurochemical changes in young rats (11). Remaining offspring (both Cu adequate and Cu deficient) were offered a nonpurified commercial diet, Purina Laboratory Chow (LRC) 5001, and tap water. The LRC diet contained 14 mg Cu/kg and 72 mg Fe/kg. One month following Cu repletion, random samples (n = 4) of Cu-adequate and Cu-repleted female and male rats were taken to evaluate recovery.

Rats were sacrificed by decapitation; livers and brains were removed, weighed, and processed for biochemical analysis. Males and females were sacrificed on consecutive days. Brains were dissected on a chilled glass plate into six parts following the guide-

Table I. Characteristics and Copper Status of 30-Day-Old Sprague-Dawley Rats

Parameter	Group			
	+CuF	−CuF	+CuM	−CuM
Body weight (g)	95 ± 2	88 ± 7	106 ± 1	93 ± 6
Brain weight (mg/g body wt)	16.7 ± 0.6	17.7 ± 1.2	15.6 ± 0.2	17.2 ± 0.9
Hemoglobin (g/l)	105 ± 0.4	109 ± 0.3	106 ± 0.3	96 ± 0.3
Ceruloplasmin (units/l)	87.8 ± 3.5	0.5 ± 0.2[a]	103 ± 3.0	0.10 ± 0.10[b]
Liver Cu (nmol/g fresh wt)	86.7 ± 14.2	26.9 ± 3.1[a]	94.4 ± 3.8	22.7 ± 3.8[b]
Liver Fe (μmol/g fresh wt)	1.16 ± 0.14	1.30 ± 0.24	1.04 ± 0.11	0.94 ± 0.20

Note. Values are means ± SEM (n = 4). Means of Cu-deficient rats were significantly different ($P < 0.05$) when tested by ANOVA for [a]females and [b]males. +CuF, Cu-adequate female; −CuF, Cu-deficient female; +CuM, Cu-adequate male; −CuM, Cu-deficient male.

lines of Glowinski and Iversen (14). The six regions were cerebellum, medulla oblongata + pons, cerebrum (cortex), hypothalamus, striatum, and midbrain + hippocampus. Regions utilized for enzymes were homogenized for 30 sec in 9 or 24 volumes of cold 0.05 *M* potassium phosphate (pH 7.0) using a Tissumizer and microprobe (SDT-080 EN; Tekmar, Cincinnati, OH).

Biochemical Analyses. Hemoglobin was determined spectrophotometrically as metcyanhemoglobin. Protein levels were measured by a modified Lowry procedure using bovine albumin as reference (15).

Livers and 1-g portions of diet were wet-digested with 4 ml of concentrated HNO_3 (AR select grade; Mallinckrodt, St. Louis, MO), and the residue was brought to 4.0 ml with 0.1 *M* HNO_3. Samples were then analyzed for total Cu and Fe by flame atomic absorption spectroscopy (model 2380; Perkin-Elmer, Norwalk, CT). The method was checked with a certified bovine liver standard (no. 1577; U.S. National Bureau of Standards and Technology, Gaithersburg, MD). Brain regions were wet-digested in 5-ml micro-Fernbach flasks by adding 1 ml of 3 *M* HNO_3 (Optima grade; Fisher Scientific, Pittsburgh, PA). The residue was suspended in 0.01 *M* 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. Accuracy was verified by analysis of primary standards (bovine liver) and by inclusion of a secondary standard, a rat whole brain digest previously analyzed by flame atomic absorption spectroscopy.

Enzyme Analyses. Plasma was obtained from microhematocrit tubes following centrifugation, and the activity of the cuproprotein ceruloplasmin (EC 1.16.3.1) was measured by following oxidation of o-dianisidine at 37°C (16). Brain homogenates were diluted in the presence of 0.1% Triton X-100 and assayed spectrophotometrically for CCO, SOD, and GPX as described in detail by Prohaska (17). CCO was assayed on fresh tissue and GPX the following day. SOD extracts were frozen at −20°C for several weeks. Activity of rat brain DBM was determined spectrophotometrically as described previously for mouse brain (18). The endogenous inhibitor of DBM activity was inactivated by 25 m*M* N-ethylmaleimide rather than copper.

Assay of PAM was performed on brain extracts by modification of the method of Katopodis and May (19). The substrate D-Tyr-Val-Gly (Sigma Chemical Co., St. Louis, MO) was derivatized by reaction with trinitrobenzene sulfonic acid and purified. The trini-

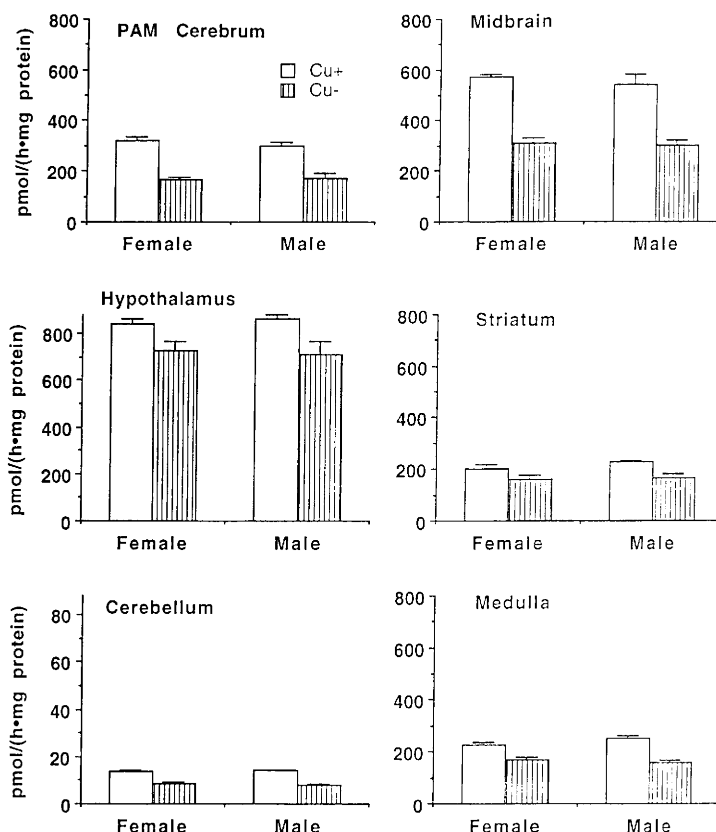

Figure 1. Effect of perinatal Cu deficiency in rats on brain regional peptidylglycine α-amidating monooxygenase (PAM) activity. Brains of month-old rats (Cu-adequate [Cu+] females [n = 4], Cu-deficient [Cu−] females [n = 4], Cu+ males [n = 4], Cu− males [n = 4]) were dissected into six regions and PAM activity was determined by HPLC. Bars represent sample means ± SEM. Means of Cu-deficient groups were lower than Cu-adequate for all regions $P < 0.01$ by ANOVA.

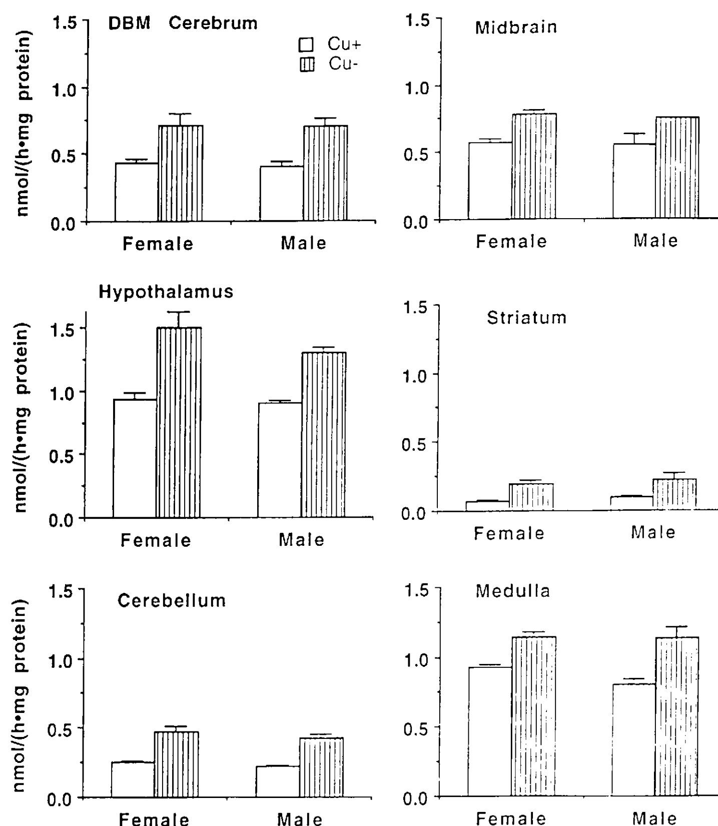

Figure 2. Effect of perinatal Cu deficiency in rats on brain regional dopamine-β-monooxygenase (DBM) activity. Brains of month-old rats (Cu-adequate [Cu+] females [n = 4], Cu-deficient [Cu−] females [n = 4], Cu+ males [n = 4], Cu− males [n = 4]) were dissected into six regions and DBM activity was determined spectrophotometrically. Bars represent sample means ± SEM. Means of Cu-deficient groups were higher than Cu-adequate for all regions $P < 0.01$ by ANOVA.

trophenyl (TNP) derivative was prepared with 92% yield and absorbed maximally at 342 nm. The TNP derivative of the PAM reaction product, TNP-D-Tyr-Val-NH_2, was also synthesized and used for quantitative analysis, molar absorptivity = 1.22×10^4 $M^{-1}cm^{-1}$ at 350 nm (20). The TNP-substrate and product were separated by reverse-phase HPLC and the effluent was monitored at 350 nm (Kratos 757 UV detector; Applied Biosystems, Foster City, CA). The mobile phase was an aqueous buffer (0.2 *M* ammonium acetate [pH 4.1], 65%; and acetonitrile [HPLC grade, Fisher Scientific], 35%). The compounds were fractionated on a 4.6 × 100 mm analytical column (ODS Spheri-5, C-18, 5 μ*M*) preceded by a 3.2 × 15 mm cartridge guard column (Aquapore ODS 7 μ*M;* Brownlee Labs, Applied Biosystems). Flow rate was 1.0 ml/min. Peak heights were recorded (Omniscribe recorder, Houston Instruments) and picomoles of product were calculated by comparison to authentic TNP-D-Tyr-Val-NH_2.

Brain homogenates were diluted in 0.005 *M* potassium phosphate (pH 7.0) containing 0.2% Triton X-100 and centrifuged at 6500*g* for 10 min. The supernates were used for enzyme assay, typically 75 μg protein in a final assay volume of 125 μl. The final reaction mixture contained the following reactants: N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) (pH 7.0), 43 m*M;* KI 25 m*M;* N-ethylmaleimide (NEM) 25 m*M;* potassium phosphate 4 m*M* (pH 7.0); ascorbic acid 2.5 m*M;* Triton X-100 0.16%; catalase 1500 units; and TNP-D-Tyr-Val Gly 9 μ*M*. The assay was run at 37°C for 2 hr (product formation is linear for 3 hr). The reaction was terminated by addition of 6 μl of 8.3 *M* TCA. Samples were placed on ice for 15 min and then centrifuged at 13,000*g* for 10 min prior to injection. The reaction mixture was found to contain 0.3 μ*M* endogenous copper, principally from ascorbate and catalase, by graphite furnace analysis.

Statistical Analysis. Data were analyzed by analysis of variance (ANOVA), and specific mean comparisons were tested by the Fisher PLSD test, α = 0.05. Brain data were analyzed by three-way ANOVA for main effects (diet, sex, and region) and for interactions. Data were analyzed using a personal computer and statistical software (Statview 4.0; Abacus Concepts, Berkeley, CA).

Results

Month-old rats used in the initial studies exhibited characteristics previously observed during Cu deficiency (Table I). Rats of both sexes had lower activity of the copper-dependent oxidase ceruloplasmin and

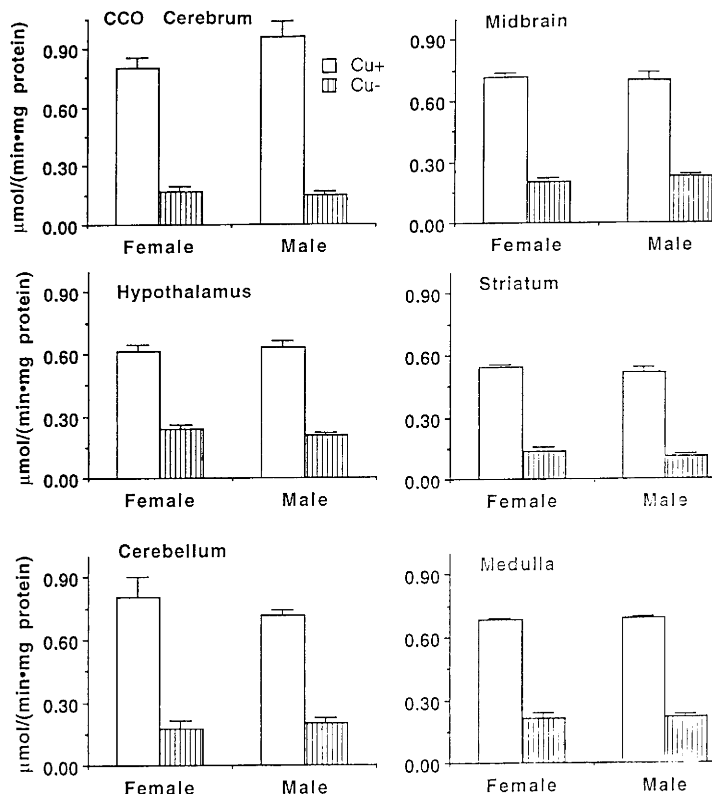

Figure 3. Effect of perinatal Cu deficiency in rats on brain regional cytochrome c oxidase (CCO) activity. Brains of month-old rats (Cu-adequate [Cu+] females [n = 4], Cu-deficient [Cu−] females [n = 4], Cu+ males [n = 4], Cu− males [n = 4]) were dissected into six regions and CCO activity was determined spectrophotometrically. Bars represent sample means ± SEM. Means of Cu-deficient groups were lower than Cu-adequate for all regions $P < 0.01$ by ANOVA.

lower concentrations of liver copper. The Cu-deficient rats, however, exhibited no statistically significant signs of growth impairment, decrease in hemoglobin levels, or elevation in liver iron concentrations (Table I). The activity of several brain enzymes were measured in six brain regions from rats of all four groups. ANOVA revealed strong effects of diet for all enzymes ($P < 0.0001$) but no diet effects on protein concentration. Sex of the rats had no effect on enzyme activity by ANOVA.

The regional levels of PAM activity varied greatly and were highest (100-fold) in the hypothalamus and lowest in the cerebellum (Fig. 1). The activity of PAM in all six brain regions was lower in samples from Cu-deficient rats of either sex compared with sex-matched Cu-adequate offspring. The percentage decrease between groups was least in the hypothalamus (13% and 18%, respectively, for females and males) and greatest in the midbrain, cortex, and cerebellum, averaging 44% ± 3%. The decrease in corpus striatum and medulla-pons PAM activity was intermediate between these extremes.

DBM activity was also greatly influenced by brain region (Fig. 2). DBM activity was highest in the hypothalamus and medulla-pons, and lowest in the corpus striatum. Activity, *in vitro,* of DBM was higher in all six brain regions studied in samples from Cu-deficient rats compared with Cu-adequate samples. The elevated DBM activity in both female and male Cu-deficient rats was least expressed, on a percentage basis, in those regions highest in absolute DBM activity.

The regional activity of CCO did not vary that much in control rats, in contrast to PAM and DBM activities. However, perinatal Cu deficiency greatly altered brain CCO activity in all six regions (Fig. 3). The percentage decrease in Cu-deficient samples ranged between 61% and 85% compared with Cu-adequate samples.

Like CCO, the activity of SOD did not differ greatly among brain regions. Dietary Cu deficiency resulted in lower SOD activity in all six regions of rats of both sexes (Fig. 4). However, the percentage decrease was least for SOD compared with the other three cuproenzymes ranging between 15% and 37% of Cu-adequate values.

The activity of a noncuproenzyme, GPX, was determined for comparison. GPX activity was similar in all six brain regions studied. Diet had only a minimal effect on GPX activity (Fig. 5). Only in cerebellum was there an effect detected statistically. The GPX activity in samples from Cu-deficient rats was higher than from Cu-adequate rats. For other regions, the GPX activity was influenced neither by diet or sex.

Other rats in the population of animals sampled at

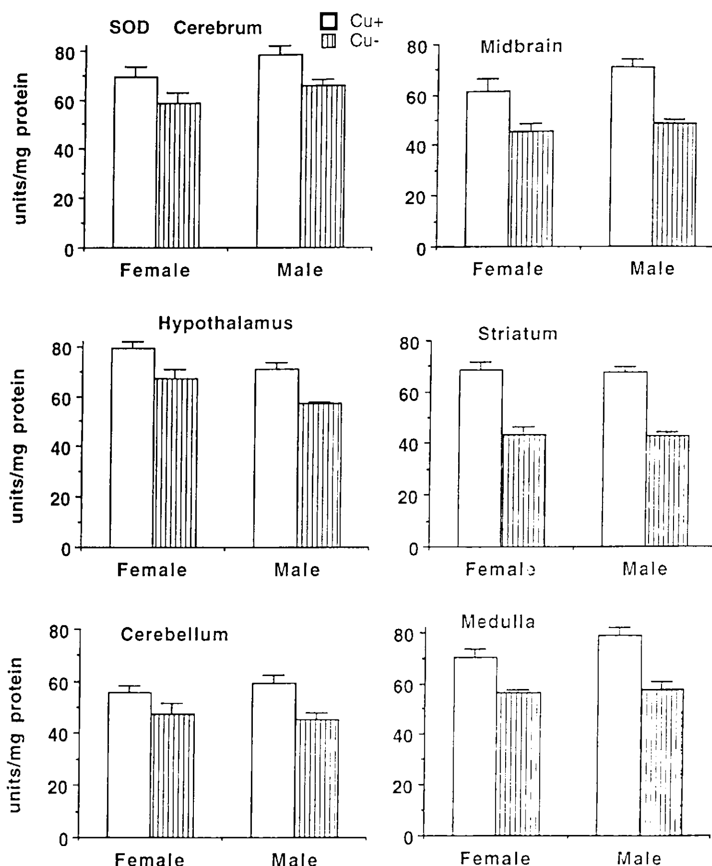

Figure 4. Effect of perinatal Cu deficiency in rats on brain regional Cu,Zn-superoxide dismutase (SOD) activity. Brains of month-old rats (Cu-adequate [Cu+] females [n = 4], Cu-deficient [Cu−] females [n = 4], Cu+ males [n = 4], Cu− males [n = 4]) were dissected into six regions and SOD activity was determined spectrophotometrically. Bars represent sample means ± SEM. Means of Cu-deficient groups were lower than Cu-adequate for all regions $P < 0.01$ by ANOVA.

age 30 days were switched to a nonpurified diet containing 14 mg Cu/kg for 1 month of dietary copper repletion. At age 2 months, there were no differences between Cu-adequate and Cu-repleted rats in body weight (males 316 g and females 212 g), ceruloplasmin activity (males 172 units/l and females 194 units/l), or liver Cu concentration (males 63 nmol/g and females 67 nmol/g). However, 1 month of repletion was not sufficient time to restore brain Cu levels (Table II). There were significant differences for both sexes of Cu-repleted rats for cerebrum, midbrain, cerebellum, and medulla. In some cases, Cu levels were still less than 50% of control values. Large sample variance was observed in the samples from hypothalamus and striatum contributing to the failure to find a difference due to treatment in those regions (Table II).

The recovery of brain enzymes following 1 month of repletion was also investigated. There were some isolated cases in which PAM activity in samples from Cu-repleted rats did not recover completely but for most comparisons there was no longer a deficit in PAM activity (Table III). The PAM activity of cerebrum for both males and females was 21% lower in Cu-repleted rats than in Cu-adequate rats. The same samples were assayed for DBM activity (Table IV). The only statistical comparison that showed higher DBM activity was the midbrain of Cu-repleted males. However, in every comparison made, the absolute value in Cu-repleted samples exceeded that of their Cu-adequate counterparts, suggesting a subtle elevation. Even after 1 month of Cu supplementation, the CCO activity of Cu-repleted rats was lower than that of Cu-adequate rats for all comparisons except the midbrain of female rats (Table V). The reduction in CCO activity in Cu-repleted rats was compared with Cu-adequate rats and was similar between regions, averaging 28% ± 7% and 36% ± 2% for females and males, respectively. The brain regional SOD and GPX activities were not different between Cu-adequate and Cu-repleted rats of either sex (data not shown). Protein concentration in brain regions was also not different between Cu-adequate and Cu-repleted rats.

Discussion

These results extend previous work on perinatal Cu deficiency in rodents (4). In particular, the observation that brain regional PAM activity is lower in Cu-deficient rats compared with Cu-adequate con-

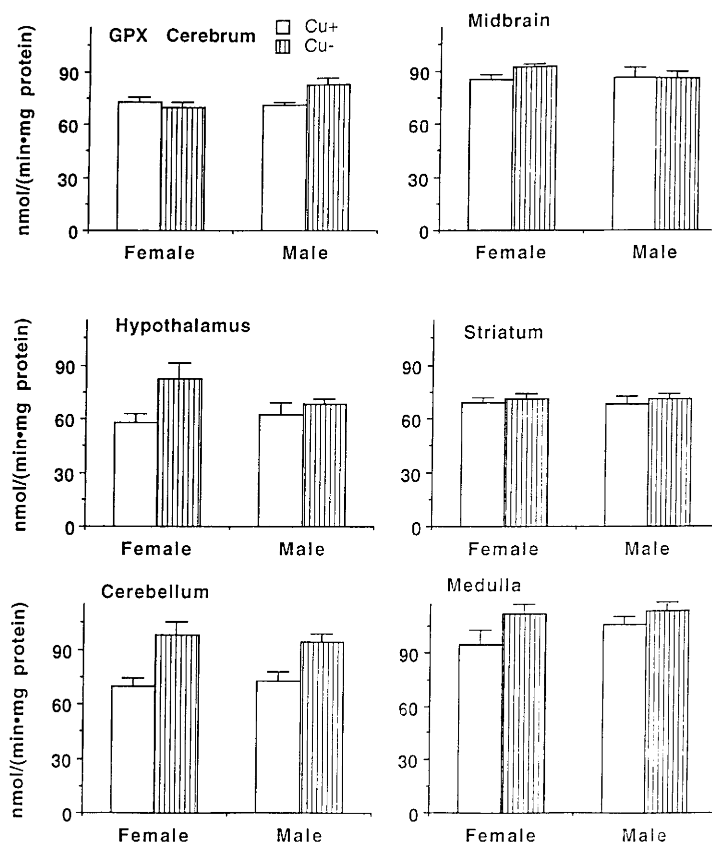

Figure 5. Effect of perinatal Cu deficiency in rats on brain regional glutathione peroxidase (GPX) activity. Brains of month-old rats (Cu-adequate [Cu+] females [n = 4], Cu-deficient [Cu−] females [n = 4], Cu+ males [n = 4], Cu− males [n = 4]) were dissected into six regions and GPX activity was determined spectrophotometrically. Bars represent sample means ± SEM. Means of Cu-deficient groups were higher than Cu-adequate for cerebellum only, $P < 0.01$ by ANOVA.

Table II. Rat Brain Regional Copper Levels Following 1-Month Copper Repletion

Region	Group (nmol/g fresh wt)			
	+CuF	rCuF	+CuM	rCuM
Cerebrum	36.2 ± 1.42	16.8 ± 0.85[a]	32.3 ± 0.45	14.7 ± 0.59[b]
Midbrain	53.1 ± 3.33	23.7 ± 2.51[a]	41.6 ± 2.11	26.4 ± 1.75[b]
Hypothalamus	60.6 ± 12.5	38.6 ± 3.69	41.0 ± 11.3	26.8 ± 0.61
Striatum	39.0 ± 2.62	28.4 ± 6.24	41.8 ± 3.17	28.8 ± 1.73[b]
Cerebellum	37.6 ± 2.46	26.7 ± 1.93[a]	34.5 ± 1.84	20.4 ± 1.19[b]
Medulla	45.8 ± 4.54	22.3 ± 1.66[a]	32.7 ± 0.92	19.2 ± 2.04[b]

Note. Values are means ± SEM of 2-month-old rats (n = 4). Means of Cu-repleted rats (rCu) were significantly different ($P < 0.05$) by ANOVA for [a]females or [b]males. Tissue copper was determined by graphite furnace atomic absorption spectroscopy after wet digestion in HNO_3. +CuF, Cu-adequate female; rCuF, Cu-repleted female; +CuM, Cu-adequate male; rCuM, Cu-repleted male.

trols, and that PAM activity, in certain brain regions, is restored slowly has not been reported previously.

Cu deficiency in the current studies did not alter brain GPX activity. The selenoenzyme GPX develops in brain during the time when dietary Cu treatments were employed in this paradigm (21). Therefore, the changes in the cuproenzymes observed in these studies were not a general reflection of undernutrition. Although Cu deficiency in rodents results in lower liver GPX activity, brain and other organ GPX activity is not altered (17).

The young month-old rats in this study exhibited features consistent with Cu deficiency. However, the degree of Cu deficiency as evaluated by body weight, liver Cu and iron levels was not as severe as in a recent study (11). This most likely is due to the slight increase in dietary Cu in the present experiments. The current Cu-deficient rats did, however, exhibit signs of Cu deficiency in the brain.

The severe drop in CCO activity is consistent with previous early observations on Cu-deficient rats (22). The modest drop in rat brain SOD activity was noted

Table III. Rat Brain Regional PAM Activity Following 1-Month Copper Repletion

Region	Group			
	+CuF	rCuF	+CuM	rCuM
	(pmol/[h · mg protein])			
Cerebrum	462 ± 18	363 ± 22[a]	391 ± 23	310 ± 9.8[b]
Midbrain	574 ± 36	643 ± 14	474 ± 54	575 ± 80
Hypothalamus	1592 ± 246	1429 ± 159	1015 ± 96	892 ± 137
Striatum	414 ± 44	371 ± 36	234 ± 22	200 ± 35
Cerebellum	15.5 ± 0.9	15.8 ± 1.4	15.9 ± 1.5	9.4 ± 0.6[b]
Medulla	388 ± 37	253 ± 22[a]	272 ± 16	239 ± 17

Note. Values are means ± SEM of 2-month-old rats ($n = 4$). Means of Cu-repleted rats (rCu) were significantly different ($P < 0.05$) by ANOVA for [a]females and [b]males. Brains were dissected into six regions and PAM activity was determined by HPLC with UV detection. +CuF, Cu-adequate female; rCuF, Cu-repleted female; +CuM, Cu-adequate male; rCuM, Cu-repleted male.

first in cerebellar extracts by Prohaska and Wells (23). That pattern seems to be consistent among all brain regions studied as shown by the current data.

The elevation *in vitro* of DBM activity in the brain of Cu-deficient rats was noted recently (11). That pattern exists for all regions examined in the current studies. This *in vitro* observation on DBM is in contrast to alterations in the pool size of norepinephrine and dopamine, the substrate and product of DBM, respectively. The original suggestion that Cu deficiency limited rat brain DBM activity was based on the measurement of lower norepinephrine levels in brains of Cu-deficient pups (23). Recent regional HPLC analysis of both catecholamines support this hypothesis (11).

The modified HPLC method for PAM activity worked well for rat brain regional analysis. The presence of NEM in the assay was essential for optimal activity (24). The method was capable of measuring PAM in regions differing in activity by 100-fold (for example, hypothalamus versus cerebellum). The brain regional differences in PAM we measured are consistent with previous reports on rats (7).

The observation that fresh tissue homogenates from brain regions of Cu-deficient rats had less apparent PAM activity than controls is novel. We expected, based on analogy of DBM, that PAM activity *in vitro* might have been elevated following Cu deficiency. This would have been consistent with earlier observations following DDC treatment (8). In that study, PAM activity estimated *in situ* was lower but *in vitro,* assayed with added Cu, was higher than control activity. Presumably, removal of Cu by chelation with DDC resulted in elevated PAM synthesis (8). The study on Cu deficiency in older rats by Mains *et al.* also assayed PAM with added Cu (10). They found higher PAM in the anterior pituitary but no change in the hypothalamus comparing Cu-deficient to control rats. We failed to detect any difference in PAM activity in midbrain homogenates of weanling rats fed a Cu-deficient diet for 5 weeks even though midbrain Cu levels had fallen 36% (24). The decrease in brain Cu reported in the Mains *et al.* study was less than 30% (10). Timing and severity of Cu deficiency influence PAM activity (24). The apparent reduction in PAM activity, *in vitro,* in samples from tissues of Cu-deficient rats suggest that PAM activity might be limiting *in vivo*. This, however, will need to be established. The *in situ* studies with Cu chelation do support this possibility. Earlier nutritional studies on ascorbic acid deficiency in guinea pig demonstrated reduction in PAM activity *in vivo* (25).

Results shown in these studies on recovery from Cu deficiency confirm earlier work in rats (26, 27) and mice (28) that showed 1 month was not sufficient to restore brain Cu levels or brain CCO activity. In fact,

Table IV. Rat Brain Regional DBM Activity Following 1-Month Copper Repletion

Region	Group			
	+CuF	rCuF	+CuM	rCuM
	(nmol/[h · mg protein])			
Cerebrum	0.68 ± 0.01	0.72 ± 0.07	0.60 ± 0.04	0.65 ± 0.02
Midbrain	0.53 ± 0.03	0.64 ± 0.10	0.42 ± 0.06	0.60 ± 0.03[a]
Hypothalamus	0.88 ± 0.05	1.04 ± 0.05	0.79 ± 0.06	0.94 ± 0.06
Striatum	0.28 ± 0.04	0.36 ± 0.04	0.21 ± 0.02	0.21 ± 0.03
Cerebellum	0.32 ± 0.02	0.37 ± 0.04	0.31 ± 0.03	0.35 ± 0.02
Medulla	0.83 ± 0.05	0.95 ± 0.02	0.75 ± 0.09	0.99 ± 0.10

Note. Values are means ± SEM of 2-month-old rats ($n = 4$). Means of Cu-repleted rats (rCu) were significantly different ($P < 0.05$) by ANOVA for [a]males. Brains were dissected into six regions and DBM activity was determined spectrophotometrically. +CuF, Cu-adequate female; rCuF, Cu-repleted female; +CuM, Cu-adequate male; rCuM, Cu-repleted male.

Table V. Rat Brain Regional CCO Activity Following 1-Month Copper Repletion

Region	Group			
	+CuF	rCuF	+CuM	rCuM
	(μmol/[min · mg protein])			
Cerebrum	1.10 ± 0.05	0.67 ± 0.04[a]	0.86 ± 0.04	0.56 ± 0.03[b]
Midbrain	0.94 ± 0.05	0.81 ± 0.19	1.02 ± 0.04	0.66 ± 0.01[b]
Hypothalamus	0.92 ± 0.04	0.71 ± 0.05[a]	1.05 ± 0.03	0.69 ± 0.02[b]
Striatum	1.05 ± 0.05	0.75 ± 0.02[a]	0.98 ± 0.02	0.59 ± 0.02[b]
Cerebellum	0.92 ± 0.03	0.72 ± 0.05[a]	1.34 ± 0.04	0.89 ± 0.03[b]
Medulla	0.77 ± 0.1	0.56 ± 0.09[a]	1.14 ± 0.09	0.74 ± 0.02[b]

Note. Values are means ± SEM of 2-month-old rats (n = 4). Means of Cu-repleted rats (rCu) were significantly different ($P < 0.05$) by ANOVA for [a]females or [b]males. Brains were dissected into six regions and CCO activity was determined spectrophotometrically. +CuF, Cu-adequate female; rCuF, Cu-repleted female; +CuM, Cu-adequate male; rCuM, Cu-repleted male.

recent studies on female rats in our lab indicate that brain Cu and CCO did not recover even after 5 months of repletion (29). SOD activity was restored following 1 month repletion in the current studies and previously (26).

Elevated DBM activity appears to fall to control levels following Cu repletion (29). Elevation of norepinephrine pool size to control levels has also been reported for both rats (26, 27) and mice (28). Thus, like SOD, the altered activity of DBM seems to reach control levels within a month of repletion.

Regional analysis of Cu levels in brain of month-old Cu-deficient offspring indicated that the hypothalamus was somewhat less affected compared with other regions (11). In the current studies, the reduction in CCO, SOD, and PAM activity in Cu-deficient rats was least in hypothalamus. The restoration of Cu content following 1-month repletion was also more evident in hypothalamus of repleted rats suggesting, in addition to the enzyme data, some regional specificity following Cu deficiency.

The long-term consequences of perinatal Cu deficiency need to be evaluated both biochemically and physiologically. The current studies do suggest widespread long-term alterations in Cu-dependent functions when nutritional Cu deficiency occurs during early development.

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1. Prohaska JR. Functions of trace elements in brain metabolism. Physiol Rev **67**:858–901, 1987.
2. Danks DM, Campbell PE, Walker-Smith J, Stevens BJ, Gillespie JM, Bloomfield J, Turner B. Menkes' kinky-hair syndrome. Lancet **1**:1100–1103, 1972.
3. Danks DM. Copper deficiency in humans. Annu Rev Nutr **8**:235–257, 1988.
4. O'Dell BL, Prohaska JR. Biochemical aspects of copper deficiency in the nervous system. In: Dreosti IE, Smith RM, Eds. Neurobiology of the Trace Elements. Clifton, NJ: Humana Press, pp 41–81, 1983.
5. Eipper BA, Mains RE, Glembotski CC. Identification in pituitary tissue of a peptide α-amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. Proc Natl Acad Sci USA **80**:5144–5148, 1983.
6. Southan C, Kruse LI. Sequence similarity between dopamine β-hydroxylase and peptide α-amidating enzyme: Evidence for a conserved catalytic domain. FEBS Lett **255**:116–120, 1989.
7. Eipper BA, Stoffers DA, Mains RE. The biosynthesis of neuropeptides: Peptide α-amidation. Annu Rev Neurosci **15**:57–85, 1992.
8. Dickinson CJ, Marino L, Yamada T. Inhibition of the α-amidation of gastrin: Effects on gastric acid secretion. Am J Physiol **258**:G810–G814, 1990.
9. Marchand JE, Hershman K, Kumar MSA, Thompson ML, Kream RM. Disulfiram administration effects substance P-like immunoreactive and monoaminergic nerual systems in rodent brain. J Biol Chem **265**:264–273, 1990.
10. Mains RE, Myers AC, Eipper BA. Hormonal, drug, and dietary factors affecting peptidyl glycine α-amidating monooxygenase activity in various tissues of the adult male rats. Endocrinology **116**:2505–2515, 1985.
11. Prohaska JR, Bailey WR. Regional specificity in alterations of rat brain copper and catecholamines following perinatal copper deficiency. J Neurochem **63**:1551–1557, 1994.
12. American Institute of Nutrition. Report of the AIN Ad Hoc Committee on standards for nutritional studies. J Nutr **107**:1340–1348, 1977.
13. American Institute of Nutrition. Second report of the AIN Ad Hoc Committee on standards for nutritional studies. J Nutr **110**:1726, 1980.
14. Glowinski J, Iversen LL. Regional studies of catecholamines in the rat brain. J Neurochem **13**:655–669, 1966.
15. Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem **87**:206–210, 1978.
16. Lehman HP, Schosinsky H, Beeler MF. Standardization of serum ceruloplasmin concentrations in international units with o-dianisidine dihydrochloride as substrate. Clin Chem **20**:1564–1567, 1974.
17. Prohaska JR. Changes in Cu, Zn-superoxide dismutase, cytochrome c oxidase, glutathione peroxidase and glutathione transferase activities in copper deficient mice and rats. J Nutr **121**:355–363, 1991.
18. Prohaska JR, Smith TL. Effect of dietary or genetic copper deficiency on brain catecholamines, trace metals and enzymes in mice and rats. J Nutr **112**:1706–1717, 1992.
19. Katapodis AG, May SW. New facile trinitrophenylated substrate for peptide α-amidation and its use to characterize PAM activity in chromaffin granules. Biochem Biophys Res Commun **151**:499–505, 1988.

20. Mokrasch LC. Use of 2,4,6-trinitrobenzenesulfonic acid for the coestimation of amines, amino acids, and proteins in mixtures. Anal Biochem **18:**64–71, 1967.
21. Prohaska JR, Ganther HE. Selenium and glutathione peroxidase in developing rat brain. J Neurochem **27:**1379–1387, 1976.
22. Gallagher CH, Judah JD, Rees KR. The biochemistry of copper deficiency. Proc R Soc London (B) **145:**134–149, 1956.
23. Prohaska JR, Wells WW. Copper deficiency in the developing rat brain: A possible model for Menkes' steely-hair disease. J Neurochem **23:**91–98, 1974.
24. Prohaska JR, Bailey WR, Lear PM. Copper deficiency alters rat peptidylglycine α-amidating monooxygenase activity. J Nutr **125:**1447–1454, 1995.
25. Fenger M, Hilsted L. Influence of ascorbic acid on in vivo amidation of alpha-melanocyte stimulating hormone in guinea pig pituitary. Acta Endocrinol **118:**119–124, 1988.
26. Prohaska JR, Wells WW. Copper deficiency in the developing rat brain: Evidence for abnormal mitochondria. J Neurochem **25:**221–228, 1975.
27. Feller DJ, O'Dell BL. Dopamine and norepinephrine in discrete areas of the copper-deficient rat brain. J Neurochem **34:**1259–1263, 1980.
28. Prohaska JR, Bailey WR. Persistent regional changes in brain copper, cuproenzymes, and catecholamines following perinatal copper deficiency in mice. J Nutr **123:**1226–1234, 1993.
29. Prohaska JR, Bailey WR. Persistent neurochemical changes following perinatal copper deficiency in rats. J Nutr Biochem **6:**275–280, 1995.