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Maternal iron supplementation attenuates the impact of perinatal copper deficiency but does not eliminate hypotriiodothyroninemia nor impaired sensorimotor development

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Abstract

Copper (Cu), iron (Fe), and iodine/thyroid hormone (TH) deficiencies disrupt brain development. Neonatal Cu deficiency causes Fe deficiency and may impact thyroidal status. One purpose of these studies was to determine the impact of improved iron status following Cu deficiency by supplementing the diet with iron. Cu deficiency was produced in pregnant Holtzman (Exp. 1) or Sprague Dawley (Exp. 2) rats using two different diets. In Exp. 2, dietary Fe content was increased from 35 to 75 mg/kg according to NRC guidelines for reproduction. Cu deficient (CuD) postnatal day 24 (P24) rats from both experiments demonstrated lower hemoglobin, serum Fe, and serum triiodothyronine (T3) concentrations. However, brain Fe was lower only in CuD P24 rats in Exp. 1. Hemoglobin and serum Fe were higher in Cu adequate (CuA) P24 rats from Exp. 2 compared to Exp. 1. Cu and TH deficient rats from Exp. 2 exhibited a similar sensorimotor functional deficit following three months of repletion. Results suggest that Cu deficiency may impact TH status independent of its impact on iron biology. Further research is needed to clarify the individual roles for Cu, Fe, and TH in brain development.

Keywords

copper deficient; rat; triiodothyronine; brain iron; sensorimotor behavior

1. Introduction

Optimal development of the mammalian central nervous system requires adequate nutritional support of a number of micronutrients [1]. Key among the many micronutrients is sufficient levels of copper, iodine, and iron. Optimal dietary iodine is necessary for psychomotor development [2]. Iodine is an essential component of thyroid hormones (TH), thyroxine (T4) and triiodothyronine (T3). TH play a key role in early brain development

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modulating the expression of a number of transcripts [3]. Adequate iron (Fe) is also widely recognized as an important essential metal for full cognitive development [4]. Likewise, perinatal copper (Cu) deficiency has been shown to impair brain development, as persistent behavioral consequences in adult rodents were detected months after Cu repletion [5, 6].

Zimmerman et al. reported that iodine alone was insufficient to treat goitrous children who were also Fe deficient [7]. This may be due to the biochemical requirement of Fe as a catalyst for thyroid peroxidase [8]. Perinatal Cu deficiency in rats results in Fe deficient pups that exhibit anemia, low serum and tissue Fe levels [9]. The neurochemical phenotype of TH, Cu, or Fe deficiency has many similarities including hypomyelination and altered energy metabolism. Recently, it was hypothesized that the abnormal brain development observed in Cu and Fe deficiency may in part be due to alteration in TH metabolism [10]. Previous work in older Cu deficient and Fe deficient rat pups reported lower serum T3 levels [11–13]. These data are also consistent with the hypothesis that Cu deficiency can result in abnormal Fe metabolism and Fe deficiency leads to impairment in TH biosynthesis.

Previously, it was shown that brain Fe levels are lower following perinatal Cu deficiency [14]. Injection of Cu deficient pups with iron dextran restored brain Fe deficits to control levels and reversed lower hemoglobin levels in Cu deficient pups [9]. Interestingly, Cu adequate pups injected with iron also displayed a rise in hemoglobin suggesting the semi-purified diet used in those studies did not contain the necessary blend of nutrients, presumably Fe, to support optimal hemoglobin building. Could extra dietary Fe also augment hemoglobin in "adequate" rodents and reverse some of the Fe deficient phenotype of Cu deficient pups?

One purpose of the current studies was to determine if increased dietary Fe could blunt the anemia of Cu deficiency and elevate tissue Fe levels. A second purpose was to compare the impact of the two semipurified copper-deficient (CuD) diets on TH status. A final objective was to test a specific behavior, vibrissae-elicited fore limb placement, previously shown to be impaired in rats who had recovered from either Fe or Cu deficiency during neonatal development [9, 15].

2. Materials and methods

2.1 Animal care and induction of copper deficiency

In Experiment One (Exp. 1), 10 Holtzman sperm-positive rats were purchased commercially (Harlan Sprague Dawley, Indianapolis, IN) and received either Cu-adequate (CuA) or Cu-deficient (CuD) dietary treatment consisting of a Cu deficient modified AIN-76A diet (Teklad Laboratories, Madison, WI) (Table 1). Normal AIN-76A diet contains approximately 6 mg Cu/kg. All dams and offspring were fed the CuD diet. CuA groups drank water supplemented with cupric sulfate, 20 mg Cu/L, and CuD groups drank Cu free deionized water. Treatment of dams began on embryonic day 7 (Table 1) [9].

In Exp. 2, 15 Sprague-Dawley sperm-positive rats were purchased commercially (Charles River, Willmington, MA) and received either CuA or CuD diets modified from the AIN-93G formulation by increasing dietary iron, copper, calcium and phosphorous to those recommended for reproduction listed in Table 2-2 of the 1995 National Research Council guidelines for rats (Table 1). CuA and CuD dams were offered deionized water to drink. Treatment of dams began on embryonic day 2. Five CuA dams were given 6-propyl-2-thiouracil (Sigma-Aldrich, St. Louis, MO), 10 mg/mL, in their drinking water on embryonic day 6 to induce hypothyroidism in their pups (THD) [10]. For both experiments one or two male pups from each litter were sampled on postnatal day 24 (P24). There was a switch from

Holtzman rats (Exp.1) to Sprague Dawley rats (Exp.2) since the latter strain was more commonly used for thyroid hormone studies.

Following termination of Exp. 2, 10 P25 female pups from each of the CuA, CuD, or THD groups were divided and placed in two cages, N=5 each. They were given tap water and non-purified laboratory chow, containing a generous supply of copper and iron (Table 1) for three months, Exp. 3. All 30 rats were weighed weekly. At the end of the study each rat was tested for their ability to respond to vibrissae-elicited forelimb placement as described previously [9].

All experiments sampled rats from a minimum of four, usually five, separate litters of each treatment group. All animals were maintained at 24°C with 55% relative humidity on a 12-h light cycle (0700–1900-h). All protocols were formally approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.2 Tissue collection

To prevent potential changes induced by anesthetics, P24 male pups were decapitated without anesthesia. Upon decapitation, trunk blood was allowed to clot and serum was harvested and stored at –75°C until analysis. An aliquot of blood was used to measure hemoglobin. Liver and brain was rapidly dissected and a portion used for metal analyses. Hearts were removed to determine the extent of cardiac hypertrophy by measurement of relative heart weight. In Exp. 2, another set of P24 pups used for metal analysis and brain T3 were anesthetized with ketamine/xylazine and intracardially perfused with phosphate buffered saline to remove blood from liver and brain.

Dams and older females were deeply anesthetized with either ketamine/xylazine (Exps. 1 and 3) or carbon dioxide (Exp. 2) prior to cardiac puncture. Plasma was harvested and an aliquot of liver and half brain used for metal analyses.

2.3 Biochemical and metabolite analyses

Diet samples, brain and liver tissue was wet-digested with HNO₃ (Trace Metal grade; Fisher Scientific, Pittsburgh, PA) and samples were analyzed for total copper, iron, and zinc content by flame atomic absorption spectroscopy (AAS) (Model 1100B, Perkin-Elmer, Norwalk, CT) [16]. Brain iron content of P24 pups in Exp. 1 was corrected for blood iron contamination [14]. Plasma or serum was analyzed for iron content by AAS following treatment with hot trichloroacetic acid as described previously [16]. Hemoglobin was determined spectrophotometrically at 540 nm after conversion to metcyanhemoglobin [9]. Diamine oxidase activity of serum ceruloplasmin was measured using o-dianisidine as substrate [17].

Serum and brain T3 levels were determined by radioimmunoassay (RIA) protocols as recently described using commercially available RIA kits (Siemens Medical Solutions Diagnostics; Los Angeles, CA) [10]. The manufacturer's RIA procedure was followed except that in-house T3 calibrators were prepared in hormone-stripped rat serum for samples in Exp. 2. Thyroid hormones were extracted from half brains using a modification of the method described by Morreale de Escobar *et al.* [18]. Brains were homogenized in methanol containing 1 µmol/mL PTU and spiked with 2 pg of ¹²⁵I-T4 tracer to determine recoveries [10].

2.4 Enzymology and western blots

Cytochrome c oxidase (CCO) activity was measured spectrophotometrically on fresh brain homogenates in Exp. 2 by monitoring oxidation of ferrocytochrome c at 550 nm [17]. Total

protein levels of brain homogenates were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford, IL).

Homogenates of P24 rat brains used for CCO assay were diluted with an equal volume of lysis buffer (0.05 M Tris, pH 8.0, 0.15 M NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% SDS) and centrifuged at 10,000xg for 5 min. A 25 µg aliquot of protein was subjected to SDS-PAGE on a 15% gel and probed for subunit IV of cytochrome c oxidase (COX IV), copper chaperone for superoxide dismutase (CCS), and lactate dehydrogenase (LDH) as a loading control. Description of the transfer, reagents used for incubation, antibodies used, and techniques of detection are described elsewhere [9]. Chemiluminescence detection and densitometry was carried out using the FluorChem™ system (Alpha Innotech, San Leandro, CA). Each COX IV and CCS value was normalized to the mean CuA value for that antigen.

2.5 Statistical analyses

Means ± SEM were calculated using Microsoft Excel™. For dams and P24 pups mean data in the four treatment groups, two diet treatments and two experiments, were analyzed by one-way ANOVA and Scheffe's test, $\alpha=0.05$. Scheffe's test was also used to evaluate mean comparisons in the three treatment groups of Exp. 3, following recovery from Cu deficiency and TH deficiency and for the three treatment groups for western blot data of Exp. 2.

3. Results

3.1 Diet comparisons

Previous work with perinatal copper deficiency using a modified AIN-76A diet suggested that the Fe content might not be sufficient to saturate tissue stores and optimize hemoglobin levels [9]. The other common rodent formulation, AIN-93G, contains the same Fe content. Thus, a modified diet was formulated as recommended by NRC guidelines to increase iron, copper, calcium, and phosphorous content while using the basic protein, fat, and carbohydrate features of the AIN-93G diet. This diet, modified AIN-93G, was analyzed for metal content along with the two CuD diets, and rodent chow used in Exp. 3 (Table 1). The two CuD diets had similar Cu contents but the modified CuA AIN-93G diet contained approximately 25% more Cu than recommended by both the AIN-76A and AIN-93G diets, which are approximately 6 mg/kg. The zinc contents of the semipurified diets were not greatly different. The metal content of the lab chow contained higher copper, iron, and zinc than the Teklad semipurified diets.

3.2 Induction of copper deficiency in rat dams and pups

Holtzman and Sprague Dawley rat dams on CuD treatment were similar in body weight following lactation (Table 2). The CuD treatment in Exp. 2 resulted in smaller dams, perhaps because the diet started at E2 rather than E7. The impact of the extra Fe in Exp. 2 is reflected in both hemoglobin and liver Fe data (Table 2). The CuD dams in Exp. 1 had lower hemoglobin levels than any other group. The liver Fe level of CuA dams in Exp. 1 was 72% lower than CuA dams of Exp. 2. Interestingly, plasma Fe was impacted in CuD dams by a similar magnitude for both experiments, 45% and 39% reductions compared to CuA values, Table 2. Copper deprivation was similar between CuD dams in both experiments as reflected by liver Cu concentration.

Body weight at P24 of the CuA and CuD pups was similar in both experiments with a significant reduction in CuD pups (Table 3). Confirming data in dams, the new modified diet with extra Fe seemed to impact both hemoglobin and liver Fe values in pups. Though both CuD groups had lower hemoglobin than their respective CuA groups, the CuA pups of

Exp. 1 had lower hemoglobin than the CuA pups of Exp. 2 (Table 3). There was much variability in the liver Fe content of the CuA pups in Exp. 2 resulting in a non-significant ANOVA. However, it seems apparent that pups in Exp. 2 had higher liver Fe content than pups of Exp. 1, similar to their dams. Liver Cu was markedly lower in CuD pups of both groups and not significantly different from one another. However, CuD pups in Exp. 2 had attenuated cardiac hypertrophy compared to the CuD pups in Exp. 1, suggesting another feature modified by the new formulation. Serum ceruloplasmin activity of CuD pups was almost non-detectable whereas activity in the CuA pups of both experiments was similar and much higher (Table 3).

3.3 Brain metal content following perinatal copper deficiency

Whole brain Cu and Fe content was measured by flame AAS (Figure 1). Pups in Exp. 2 were perfused to remove metal contamination from blood. Perinatal Cu deficiency resulted in similar marked reduction in P24 brain Cu contents in CuD pups of both experiments, 81% and 76% respectively. Interestingly, the brain Fe data revealed that the CuD pups raised by dams on the new modified diet no longer experienced a reduction in brain Fe (Figure 1). The CuD pups in Exp. 1 had a 35% reduction in brain Fe content. Brain Cu and Fe content of the P24 thyroid hormone deficient (THD) pups of Exp. 2 were similar to CuA values (data not shown).

3.4 Serum iron and T3 levels following perinatal copper deficiency

The RIA method for determining serum T3 concentrations was slightly different for Exps. 1 and 2 in that human versus rat serum calibrators were used, respectively. Thus, the absolute values for the two experiments differed significantly (Figure 2). Importantly though, in both experiments there was a significant reduction in serum total T3 in CuD pups, 47% and 30%, respectively. The mean serum T3 concentration of THD pups was 0.083 pmol/mL, 91% lower than the CuA pups. Serum T4 concentration was not impacted by Cu deficiency when evaluated in Exp. 2; however, the concentration in THD pups were lower by 88% compared to CuA pups (data not shown).

Brain T3 was also significantly lower by 90% in the THD pups, 0.51 ± 0.06 ng/g, compared to CuA pups, 5.31 ± 0.29 ng/g, whereas CuD pups had no significant reduction in brain T3 averaging 4.96 ± 0.23 ng/g, $P > 0.05$.

Serum Fe was measured in pups for both experiments by the same method (Figure 2). P24 CuD pups had lower serum iron compared to CuA pups in both experiments, 63% and 77% reductions, respectively. The serum Fe level of the CuA pups in Exp. 2 was noticeably higher than the CuA pups of Exp. 1 (Figure 2). This was not true for brain Fe in which both CuA groups had similar concentrations (Figure 1).

3.5 Brain cuproproteins following perinatal copper deficiency

Brains of P24 CuA, CuD, and THD male pups were assayed for cytochrome c oxidase (CCO) activity, a cuproenzyme known to be markedly impacted by both dietary Cu deficiency and thyroid hormone insufficiency [19, 20]. CCO activity in the CuD samples was 72% lower than CuA controls, 0.112 ± 0.007 units/mg compared to 0.402 ± 0.019 , $P < 0.01$. CCO activity in the THD pups was not different than CuA values, 0.363 ± 0.011 . However, when the content of subunit IV of CCO was determined by western blot in the same samples there was significantly lower abundance for both CuD and THD pups (Figure 3), $P < 0.05$. The reduction in THD samples was more modest, 40%, compared to 85% lower abundance for COX IV in CuD samples. The COX IV result for THD samples was likely not directly due to a Cu deficit as the abundance of copper chaperone for superoxide

dismutase (CCS) was equivalent in CuA and THD samples (Figure 3). There was a robust 3.8-fold higher CCS abundance in CuD samples, as expected [21].

3.6 Recovery from perinatal copper deficiency

Female P25 pups (CuA, CuD, and THD) began recovery treatment drinking tap water and consuming non-purified rodent chow that contained adequate Cu and Fe (Table 1). The average body weights at the start of the repletion reflected prior treatments: CuA 74g, CuD 59g, and THD 43g. Growth was monitored over the three month recovery period and when killed these body weight differences were no longer evident (Figure 4). Even the former THD rats had body weights equivalent to CuA controls. The repleted CuD group (CuDR) actually weighed more than the THDR group. Interestingly, the three month repletion was not sufficient to restore brain Cu levels in the CuDR group to control levels (Figure 4) emphasizing the importance of brain metal accretion during early development. Plasma Fe differences were no longer evident in the CuDR group (Figure 4). Other characteristics seen in CuD pups at P24 (Table 3) were also absent, including recovery of low hemoglobin and liver Cu levels (data not shown). Brain Fe was measured and no significant differences between groups was detected, similar to data at P24 in Exp. 2 (Figure 1).

To evaluate sensorimotor development following recovery from perinatal Cu deficiency and TH deficiency, vibrissae-elicited forelimb placement was measured in 10 rats of each treatment group, Exp. 3, the day prior to killing (Figure 4). An impaired response was observed in both CuDR and THDR groups compared to CuA controls. Interestingly, the magnitude of the impairment was similar between the two former deficient treatment groups despite vast differences in brain T3 at the onset of repletion based on data of their brothers.

4. Discussion

Modification of the commonly used AIN-93G formulation to NRC recommendations for Cu, Fe, calcium and phosphorus resulted in detectable changes in Fe status especially increased liver Fe in both CuA pups and dams when compared to rats reared on modified AIN-76A diet that has mineral levels of iron similar to the current AIN-93G formulation. There was also detectably higher hemoglobin in CuA dams and pups when dietary Fe was increased from approximately 50 mg/kg to 80 mg/kg. It was somewhat surprising that the CuD dams in Exp.2 were not anemic, as diets low in copper fed during gestation and lactation usually lead to lower hemoglobin levels [22]. It may be that the response of the rats to the modified AIN-93G diet was dependent on strain. Others have shown subtle behavioral differences between Holtzman and Sprague Dawley rats [23]. Importantly, Sprague Dawley rats fed the modified AIN-76A formulation used in Exp. 1 do develop anemia suggesting that diet composition not strain of rats is responsible for the attenuation in hemoglobin levels in Exp. 2 [17].

The usual practice of adding only 35 mg/kg Fe to the AIN formulations may not be sufficient during this perinatal period. Importantly, this extra Fe added to diets in Exp. 2 reversed the brain Fe deficit seen in CuD rat pups at P24 in Exp. 1 and previously [14]. Despite brain Fe levels equivalent to CuA values in these CuD pups, there were clear signs consistent with Cu deficiency such as reduction in brain Cu, lower CCO activity and protein, and augmented levels of brain CCS; features expressed by CuD rats fed a diet similar to Exp. 1 [24].

Many mechanisms have been proposed to explain abnormal brain development and behavior following Cu deficiency [25]. These include changes in cuproenzymes such as dopamine- β -monooxygenase that limits norepinephrine synthesis [19]. Brain superoxide dismutase activity is lower following Cu deficiency [19]. This might contribute to the aberrant reactive

oxygen species (ROS) hypothesis [25]. Cytochrome c oxidase activity, heme *a* content, and protein level are all markedly lower in Cu deficient rat brain [24, 26]. This might explain the abnormal energy hypothesis and account for disturbances in mitochondrial metabolism and altered glycolysis [27]. Another cuproenzyme that may be involved is peptidylglycine α -amidating monooxygenase (PAM) whose activity in brain of CuD rats is lower following Cu deficiency [28]. A recent study suggested that the brain behavioral phenotype of PAM +/- mice was similar to wild-type mice that were marginally Cu deficient [29]. There certainly are other theories consistent with secondary factors that may impact brain development such as impaired neurite outgrowth, activation of NMDA-type glutamate receptors, and aberrant nitric oxide metabolism, reviewed elsewhere [25].

Results of current studies and recent endeavors suggest an additional possibility—abnormal TH metabolism. Rats reared by dams treated with PTU had greatly diminished serum and brain T3 levels compared to CuA or CuD pups. However, following recovery the former TH deficient pups impaired behavior was equivalent to the persistent impaired behavior of former CuD rats. This does not prove that the abnormal behavior of the repleted CuD rats was due to TH deficiency during development but does suggest the perinatal Cu deficiency can result in behavioral changes as robust as those imposed by severe hypothyroidism. Further, since these CuD rats had normal brain Fe prior to and following repletion it seems unlikely that secondary Fe deficiency is a likely cause of the brain-dependent phenotype of CuD. Current metal and behavioral data and that generated by proton NMR evaluation of CuD rat brain suggest that Fe limitation cannot fully explain the neuropathological consequences of Cu deficiency [30].

Following dietary Cu deficiency rat pups of two different strains fed two different CuD diets both exhibited significant reductions in serum T3. These data are consistent with previous reports that Cu deficient rats have lower serum T3 levels [10, 12]. Does this reduction in steady state T3 levels functionally affect brain development? This is difficult to answer currently, but a trend for reduction in several TH-dependent transcripts in whole brain of P12 CuD pups is consistent with this possibility [10]. Furthermore, the functional effects of hypothyroidism are more severe in conjugation with dietary Cu deficiency [31]. These results support the hypothesis that at least some of the known effects of copper deficiency on the developing brain may be mediated through copper-dependent reductions in T3 levels.

The molecular mechanisms responsible for lower serum T3 levels in Cu deficient rats are not precisely known however several alternate hypotheses have been proposed. First, induction of a secondary Fe deficiency in the thyroid may limit activity of thyroid peroxidase [8]. Serum Fe was lower in P24 CuD rats for both experiments in this study. Although elevating dietary Fe in Exp. 2 eliminated the reduction in brain Fe in CuD rats, direct assessment of thyroid peroxidase activity in thyroidal tissue will be needed to ascertain if activity of this enzyme is impacted by Cu deficiency.

A second possibility for lower T3 in CuD rats is alteration of the hypothalamic-pituitary-thyroid axis. Synthesis of active thyrotropin-releasing hormone (TRH) requires the cuproenzyme PAM [32]. Cu deficiency is associated with lower enzyme activity of PAM [33]. Perhaps limiting PAM activity may blunt TH biosynthesis impairing the pituitary response to TRH. Importantly, PAM heterozygote mice (PAM +/-) were recently shown to possess an impaired thyroid axis and this impairment was reversed by Cu supplementation [34]. Further, the T4 response to injected TRH was shown to be abnormal in Cu deficient rats [35].

A third possibility is that conversion of T4 to T3 by the selenoenzymes, Type I or Type II 5'-deiodinase, is limiting in Cu deficiency. It is known that activity of certain selenoenzymes is

lower in the liver of Cu deficient rats [12, 36]. Liver 5'-deiodinase activity was reported to be lower in one study and not altered in another, thus further research on this hypothesis is needed [13, 37].

Current data imply that generous supply of dietary Fe during development can eliminate some but not all of the signs of Fe deficiency in CuD pups. For example, CuD pups are still anemic and have lower serum Fe levels. It is not hard to imagine a scenario in which young pregnant women might consume a diet marginal in Cu, low in Fe and iodine during pregnancy and lactation. The interaction of this micronutrient paradigm imbalance will be interesting to explore to determine the extent that dietary Cu and Fe impact TH biology and thus human development.

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Figure 1.

Brain metal analyses following perinatal Cu deficiency in P24 male Holtzman rat pups (Exp. 1) or Sprague Dawley pups (Exp. 2). Metal content was determined by flame AAS based on wet weight. Bars represent means $\pm$ sem (n=4 or 5) for CuA, open bars, or CuD, shaded bars. Data were analyzed by ANOVA and Scheffe's test. Means with unlike superscripts are different, $P < 0.05$.

Figure 2.

Serum triiodothyronine (T3) and serum Fe levels following perinatal Cu deficiency in P24 male Holtzman rat pups (Exp.1) or Sprague Dawley pups (Exp. 2). Bars represent means $\pm$ sem (n=5–10) for CuA, open bars, or CuD, shaded bars. Data were analyzed by ANOVA and Scheffe's test. Means with unlike superscripts are different, $P < 0.05$.

Figure 3. Brain proteins following perinatal Cu deficiency or thyroid hormone deficiency in P24 Sprague Dawley rats. (A) Western blots were performed on brain homogenates for brain COX IV, CCS, and LDH using 25 μ g protein on 15% gels. (B) Quantification of densities of COX IV and CCS relative to CuA mean. Bars represent means $\pm$ sem (n=4) for CuA, open bars, CuD, shaded bars, THD, solid bars. Data were analyzed by ANOVA and Scheffe's test. Means with unlike superscripts are different, $P < 0.05$.

Figure 4.

Body weight, brain Cu, plasma Fe, and response to vibrissae-elicited forelimb placement were measured in female Sprague Dawley rats from Exp. 2 three months following recovery from perinatal Cu deficiency (CuDR), drug-induced thyroid hormone deficiency (THDR) or in controls (CuA). Cu content was determined by flame AAS and was based on wet weight. A score of 20 (10 right and 10 left trials) on the vibrissae-elicited placement test represents a perfect response. Bars represent means $\pm$ sem (n=10 or n=5 metals). Data were analyzed by ANOVA and Scheffe's test. Means with unlike superscripts are different, $P < 0.05$.

Table 1

Metal content of rat diets

Characteristic	Experiment One	Experiment Two		Experiment Three
Short Name	CuD	CuD	CuA	Lab Chow
Product ID	TD 80388	TD 08585	TD 08584	5001
Formulation	Modified AIN-76A	Modified AIN-93G	Modified AIN-93G	Nonpurified
Copper mg/kg	0.36	0.46	8.73	11.8
Iron mg/kg	50.2	78.3	80.3	174
Zinc mg/kg	47.8	43.1	41.1	78.6
Onset of Treatment	Embryonic day 7	Embryonic day 2	Embryonic day 2	Postnatal day 25

Metals were analyzed by flame AAS following wet digestion. Values are means of triplicate samples. Diets were purchased from Harlan (Teklad Custom Diets) or Ralston Purina. CuA rats in Exp. 1 were offered the CuD diet and copper-supplemented drinking water, 20 mg Cu/L.

Table 2

Characteristics of Holtzman and Sprague Dawley rat dams

Characteristic	Experiment One		Experiment Two	
	CuA	CuD	CuA	CuD
Body Weight (g)	324 ± 7.2 ^a	334 ± 7.3 ^a	319 ± 14 ^{ab}	275 ± 10 ^b
Hemoglobin (g/L)	173 ± 3 ^a	123 ± 15 ^b	187 ± 3 ^a	174 ± 4 ^a
Liver Cu (nmol/g)	65.5 ± 5.4 ^a	14.0 ± 4.4 ^b	56.5 ± 1.6 ^a	16.9 ± 4.0 ^b
Liver Fe (μmol/g)	0.92 ± 0.15 ^b	2.5 ± 0.44 ^{ab}	3.30 ± 0.43 ^a	2.64 ± 0.65 ^{ab}
Plasma Fe (nmol/mL)	86.8 ± 3.6 ^a	47.8 ± 4.9 ^b	89.0 ± 16.8 ^a	54.7 ± 1.2 ^b

Values are means ± SEM (n=5). Holtzman rats were used in Experiment One and Sprague Dawley rats in Experiment Two. Data were analyzed by ANOVA and Scheffe's test. Means with unlike superscripts are different, P < 0.05.

Table 3

Characteristics of P24 male rat pups

Characteristic	Experiment One		Experiment Two	
	CuA	CuD	CuA	CuD
Body Weight (g)	76.9 ± 1.1 ^a	58.8 ± 2.3 ^b	73.5 ± 2.2 ^a	59.2 ± 2.9 ^b
Hemoglobin (g/L)	112 ± 5.9 ^b	70.9 ± 2.1 ^d	131 ± 2.6 ^a	87.1 ± 1.5 ^c
Liver Cu (nmol/g)	96.8 ± 14.0 ^b	6.62 ± 0.27 ^c	147 ± 12.2 ^a	9.84 ± 0.27 ^c
Liver Fe (μmol/g)	0.38 ± 0.02	0.65 ± 0.09	0.93 ± 0.29	0.99 ± 0.07
Heart/BW (mg/g)	5.12 ± 0.14 ^c	12.8 ± 0.64 ^a	5.30 ± 0.09 ^c	9.21 ± 0.26 ^b
Ceruloplasmin (U/L)	86.7 ± 3.7 ^a	0.85 ± 0.62 ^b	98.5 ± 5.3 ^a	< 0.3 ^b

Values are means ± SEM (n=5–10). Holtzman rats were used in Experiment One and Sprague Dawley rats in Experiment Two. Data were analyzed by ANOVA and Scheffe's test. Means with unlike superscripts are different, $P < 0.05$.