Published in final edited form as:

Brain Res. 2008 April 14; 1204: 69–76.

Copper deficiency results in AMP-activated protein kinase activation and acetylCoA carboxylase phosphorylation in rat cerebellum

Anna A. Gybina and Joseph R. Prohaska

Department of Biochemistry and Molecular Biology, University of Minnesota Medical School, Duluth USA

Abstract

Copper (Cu) deficiency impairs cerebellar development including biosynthetic processes like myelination and synaptogenesis. The activity of cerebellar mitochondrial cuproenzyme cytochrome c oxidase is markedly lower in Cu deficient rat pups and is accompanied by higher lactate levels indicating mitochondrial inhibition. Cu deficiency impaired energy metabolism is thought to contribute to developmental delays, but specific mechanisms linking these phenomena have remained unexplored. AMP activated protein kinase (AMPK) is a cellular energy sensor that is activated during mitochondrial inhibition and shuts down biosynthetic processes to help conserve cellular ATP levels. Activated AMPK phosphorylates and inhibits acetyl CoA carboxylase (ACC), the first enzyme in fatty acid biosynthesis. We hypothesize AMPK is activated and ACC inhibited in Cu deficient cerebella. Perinatal copper deficiency was studied in young rats in rapidly frozen cerebella. Compared to copper adequate (Cu⁺) pups, copper deficient (Cu[−]) pups were hypothermic, had lower brain copper levels and markedly higher cerebellar lactate. Concentration of phosphorylated AMPK (pAMPK), indicating AMPK activation, was robustly higher in Cu[−] cerebella of rat pups at two ages and in two separate experiments. Compared to Cu⁺ cerebella, pACC content was significantly higher in all Cu[−] samples. Mechanisms leading to AMPK activation remain elusive. Higher AMP/ATP ratios and increased reactive nitrogen species (RNS) can lead to AMPK activation. ATP and AMP concentrations were unaltered and nitric oxide metabolites and 3-nitrotyrosine peptide levels remained unchanged in Cu[−] cerebella. AMPK activation may explain how ATP levels can be maintained even with a severe mitochondrial loss of CCO function.

Keywords

copper deficient; AMP activated protein kinase; acetylCoA carboxylase; 3-nitrotyrosine; ATP; lactate

1. Introduction

Copper (Cu) is a necessary nutrient for proper mammalian development. In vivo Cu functions primarily as a co-factor for several enzymes (cuproenzymes) including superoxide dismutase, ceruloplasmin, and dopamine β-monooxygenase. Shortly after the essentiality of Cu was

Address correspondence to: Joseph R. Prohaska, Department of Biochemistry and Molecular Biology, University of Minnesota Medical School Duluth, 1035 University Drive, Duluth, MN 55812, Phone: 218 726-7502, Fax: 218 726-8014, E-mail: jprohask@d.umn.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

established in rats it was shown that another cuproenzyme, cytochrome *c* oxidase (CCO) was particularly sensitive to Cu deficiency (Cohen and Elvehjem, 1934). As a crucial component of the electron transport chain, low CCO activity can impair mitochondrial function, which can have serious ramifications for the energy-demanding brain. Cu deficiency is a hallmark of Menkes disease, which stems from deletion of the Cu transport protein ATP7A (Danks et al., 1972; Mercer, 1998). Reduced CCO activity is a key characteristic of this human neurodegenerative disease. Neurological manifestations of Menkes disease include severe mental retardation and defective brain development, particularly in the cerebellum (Menkes et al., 1962).

Neurological manifestations seen in Menkes disease are also seen in dietary copper deficiency in rats (Prohaska and Wells, 1974). Assessments of cerebellar function indicate persistent sensory motor abnormalities in rats following perinatal copper deficiency (Penland and Prohaska, 2004; Prohaska and Hoffman, 1996). This may be due to the fact that perinatal Cu deficiency blunts cerebellar growth (Everson et al., 1967; Gybina and Prohaska, 2003; Prohaska and Wells, 1974) and limits biosynthetic processes like myelination and synaptogenesis (El Meskini et al., 2007; Prohaska and Wells, 1974; Prohaska, 1981; Zimmerman et al., 1976); however, the precise mechanisms responsible remain poorly understood.

Though it does not provide a direct explanation, Cu deficiency impaired energy metabolism has been repeatedly suggested as a cause for developmental delay in Cu[−] rats. Past work has indicated that chronic Cu deficiency impairs brain mitochondria. Chronic Cu deficiency in brain reduces CCO activity, heme *a* content, and CCO protein levels (Gybina and Prohaska, 2006; Prohaska and Wells, 1974; Prohaska and Wells, 1975). Transmission electron micrographs of Cu deficient rat brains show large, distended, vacuolized mitochondria (Prohaska and Wells, 1975). Ultimately, Cu deficiency leads to mitochondrial impairment, reflected by markedly elevated brain lactate levels in Cu[−] rats. However, what may link impaired energy metabolism to decreased biosynthesis in brain has previously been unclear.

Recent research alludes to a potential link. AMP activated protein kinase (AMPK) is an energy homeostatic sensor that is activated by energetic deprivation occurring during anoxia and mitochondrial inhibition and functions to reduce energy expenditure and augment ATP generating processes in order to sustain cellular energy levels (Kahn et al., 2005). Previous studies have shown AMPK to be an important molecule to the survival of neurons and other brain cells during mitochondrial inhibition and glucose deprivation (Almeida et al., 2004; Blazquez et al., 1999; Culmsee et al., 2001). Importantly, activated AMPK shuts off biosynthetic pathways like fatty acid synthesis by inhibiting the first enzyme in this pathway – acetyl CoA carboxylase (ACC).

Current experiments used a well characterized model of perinatal Cu deficiency to induce mitochondrial inhibition. These experiments were designed to test the hypothesis that AMPK is activated in the cerebellum under Cu deficient conditions and this activation leads to the inhibition of ACC. Subsequent effects on cerebellar adenine nucleotide concentrations and reactive nitrogen species (RNS) were evaluated to determine energy status and possible mechanisms for AMPK activation.

2. Results

2.1 Characterization of Cu deficiency in rats and cerebellar extraction

Cu deficiency at P13 and P24 rats was evidenced by reduced brain Cu concentrations at both ages and slowed growth that was apparent in Cu[−] animals at P24. Two sets of experiments were run to determine biological reproducibility of our results; the degree of brain Cu

deficiency and differences in body weights were similar in both experiments. In Experiment I P13 pups of both treatments weighed approximately 33g. At P24 Cu+ pups averaged 80 ± 4 g (means $\pm$ SEM) compared to 56.3 ± 3 g for Cu- pups. Similar weights were observed at P24 in Experiment II (data not shown). Brain Cu levels in Cu+ P13 pups averaged 1.31 ± 0.06 μ g/g compared to 0.37 ± 0.02 for Cu- pups, a 72% reduction. At P24 the reduction was more severe, 83% for Experiment I and 81% for Experiment II, $P < 0.01$. For Experiment I P24 Cu+ pups had brain Cu values of 2.10 ± 0.03 μ g/g compared to 0.36 ± 0.02 for Cu-. For Experiment II P24 Cu+ pups had brain Cu values of 2.30 ± 0.06 compared to Cu- values of 0.44 ± 0.04 . Copper deficiency induced for the purposes of this study closely parallels the degree and manifestation of deficiency induced in previous work (Gybina and Prohaska, 2003; Gybina and Prohaska, 2006; Prohaska and Wells, 1975).

Following decapitation the cerebellum was rapidly removed from Cu+ and Cu- animals to study the effect of Cu deficiency on AMPK activation. The anatomical location of the cerebellum in the posterior of the skull allows for rapid extraction and freezing of this brain region. Rapid tissue inactivation minimizes anoxic AMPK activation, which might otherwise mask potential activation in the Cu deficient cerebellum. Rapid tissue freezing also allowed for determination of metabolite levels reflecting near in vivo conditions. There were no significant differences between Cu+ and Cu- cerebellar extraction times (see experimental procedures).

2.2 Characterization of cerebellar mitochondrial inhibition due to Cu deficiency

The extent of cerebellar mitochondrial dysfunction induced by Cu deficiency was characterized. Analysis of fast-frozen P13 and P24 cerebella confirmed that Cu deficiency markedly reduced CCO activity (more than 75%) and led to chronic inhibition of cerebellar mitochondria evidenced by higher than control cerebellar lactate concentrations throughout the first month of development (Fig. 1A). In Experiment I, at both ages, Cu- rat pup absolute CCO activity (μ mol/min $\times$ mg) was significantly lower than the age matched controls (0.056 ± 0.038 vs 0.209 ± 0.003 at P13 and 0.049 ± 0.037 vs 0.390 ± 0.033 at P24 ($n=4$)). Experiment I cerebellar lactate concentration (μ mol/g) was markedly higher in Cu- rats (2.71 ± 0.26 vs 1.56 ± 0.19 at P13 and 6.49 ± 0.69 vs 2.59 ± 0.50 at P24 ($n=4$)). Relative to age matched Cu+ pups, P13 and P24 Cu- cerebellar CCO activity was 73% and 88% lower, respectively, while lactate concentrations were 75% and 150% higher. This suggested an age dependent progression in the severity of mitochondrial inhibition (Fig. 1A). For both Experiment I and II, P24 cerebellar lactate of Cu- rats was markedly elevated confirming a similar Cu- metabolic stress (Fig. 1A and 1B). For Experiment II Cu- cerebellar lactate was 6.05 ± 0.32 μ mol/g compared to Cu+ values of 1.86 ± 0.04 , $P < 0.01$.

Hypothermia has been documented in Menkes patients, but has not previously been assessed in dietary Cu deficiency (Mercer, 1998). Body temperature of unanesthetized rats was measured as an additional assessment of over-all mitochondrial impairment. Compared to Cu+ controls highly significant hypothermia in Cu- P24 rats was detected (Fig. 1B) (36.4 ± 0.2 vs 37.3 ± 0.1 ($^{\circ}$ C) ($n=5$) $P < 0.01$).

2.3 AMPK is activated in Cu- cerebella

AMPK is activated by phosphorylation on threonine 172 of the $\alpha 1$ and $\alpha 2$ subunits. To test the hypothesis that AMPK is activated in Cu- cerebella experiencing mitochondrial inhibition, phosphorylation and total protein levels of AMPK in Cu- and Cu+ rat cerebella were analyzed. Western blot analysis determined that Cu- rat cerebella have significantly higher content of activated AMPK (Fig. 2). Analysis of both P13 and P24 fast-frozen cerebella (Experiment I) revealed significantly higher levels of phosphorylated AMPK (pAMPK) in Cu- rats as compared to Cu+ controls. The elevation of pAMPK in Cu- cerebella was more notable at

P24, 130% higher, compared to P13, which was 36% higher. Total AMPK levels, however, were not altered by Cu deficiency at either age. These results were confirmed in Experiment II (n=4 per group), which also showed significantly higher pAMPK levels in Cu- cerebella without changes to total AMPK levels ($P < 0.05$) (data not shown). Cu deficiency status of the samples was confirmed by stripping the western blot membranes previously used for AMPK analysis and probing for the Cu deficiency marker copper chaperone for superoxide dismutase (CCS). CCS protein levels are higher following Cu deficiency (Bertinato et al., 2003; Prohaska et al., 2003). CCS levels were markedly higher in Cu- cerebellar extracts and confirmed the Cu deficient status of the samples used (Fig. 2). At P13 the CCS density of Cu- samples was 39% higher and at P24 the CCS density of Cu- samples was 94% higher, in rough agreement with the more severe reduction in brain Cu levels at P24 compared to P13 and similar to the qualitative change in pAMPK density in Cu- samples at the two ages.

2.4 ACC is inhibited in Cu- Cerebella

Given AMPK activation, we determined whether it resulted in a change to the phosphorylation state of AMPK's in vivo target protein acetylCoA carboxylase (ACC) (Fig. 3). Western blot analysis used an antibody specific to phosphorylation of serine 79, the AMPK specific ACC target residue (Brownsey et al., 2006; Munday, 2002). Evaluation of both P13 and P24 cerebella (Experiment I) revealed higher content of phosphorylated ACC (pACC) in Cu- cerebella compared to age matched Cu+ controls, 94% and 61%, respectively. Equal loading of lanes was visualized with Ponceau S staining. Brain ACC exists as one of two isoforms both of which are visible on the western blots. P13 levels of pACC are more pronounced as expression of ACC in rodent brain decreases as the animal approaches weaning (Spencer et al., 1993). Perhaps this is why the impact of Cu deficiency at P13 was more pronounced than at P24. P24 results, however, were further confirmed in Experiment II ($P < 0.05$, n=4) (data not shown). Together these data verify that AMPK is activated by chronic Cu deficiency in cerebellum and acts in vivo to phosphorylate its physiological target ACC.

2.5 Cerebellar nucleotide levels are unaltered by Cu deficiency

One mechanism leading to AMPK activation is the elevation in the AMP/ATP ratio. Thus, impact of chronic mitochondrial inhibition on concentrations of ATP and AMP in Cu- cerebella was analyzed at P13 and P24 and compared to Cu+ controls. Analysis of P13 rats from Experiment I and P24 rats from Experiment II showed that, despite evidence of mitochondrial inhibition (considerably higher lactate levels), neither ATP nor AMP concentrations were significantly altered as compared to Cu+ cerebella at either age (Table 1). Furthermore, determination of the AMP/ATP ratio also yielded no significant differences between Cu+ and Cu- rat cerebella.

2.6 Cerebellar nitric oxide metabolism is unaltered by Cu deficiency

Another potential mechanism leading to AMPK activation in Cu- cerebellum was evaluated. Others have shown that enhanced reactive nitrogen species (RNS) can elevate levels of pAMPK (Zou et al., 2004). Peroxynitrite levels, evaluated by detection of protein 3- nitrotyrosine levels, were not augmented by Cu deficiency in cerebellum (Fig. 4A). Analysis of cerebellar homogenates by western blot detected only one 30 kDa 3-nitrotyrosine positive band. There may have been changes in less abundant peptides not detected. Densities of the 30 kDa western blot band in Cu- cerebellar samples were 1.12 ± 0.08 (n=4) compared to 1.00 ± 0.08 in the Cu + cerebellar controls ($P > 0.05$). Though no changes in 3-nitrotyrosine levels were detected, there was good evidence of severe Cu deficiency in these Cu- cerebella, as we detected a robust augmentation of CCS levels and there was a marked reduction in CCO subunit IV (COX IV). Actin levels were equivalent between Cu- and Cu+ samples (Fig. 4A).

Nitric oxide (NO) metabolite levels, nitrite and nitrate, were measured spectrophotometrically with the Griess reagent and were unaltered in cerebellar extracts of Cu⁻ pups compared to Cu⁺ pups (Fig. 4B). Furthermore, plasma NO metabolite levels were also not altered by Cu deficiency. Levels in Cu⁻ rat pups averaged 55.1 ± 2.2 (n=4) nmol/mL compared to 59.1 ± 1.3 (n=3) for Cu⁺ pups, $P > 0.05$.

3. Discussion

Cu is an essential micronutrient and a key cofactor for CCO activity and assembly (Shoubridge, 2001). Cu deficiency in brain leads to extensive mitochondrial inhibition and structural abnormalities (Gybina and Prohaska, 2006; Prohaska and Wells, 1974; Prohaska and Wells, 1975). The resulting impaired energy metabolism may be responsible for limited biosynthesis in Cu⁻ cerebella. AMPK is a cellular energy sensor that is activated by energetic stress and acts to shut off energy expenditure on biosynthetic processes like fatty acid synthesis by inhibiting ACC. We hypothesized that Cu⁻ cerebellum experiences AMPK activation and the phosphorylation of its target protein ACC. Employing a well characterized rat model of dietary Cu deficiency, mitochondrial inhibition was induced and characterized in the cerebella of young rats over a period of weeks (Gybina and Prohaska, 2003; Gybina and Prohaska, 2006; Prohaska and Wells, 1975).

Our current experiments extended previous studies of mitochondrial dysfunction in Cu deficiency and found that Cu⁻ cerebellar mitochondria were increasingly impaired during development as evidenced by lower CCO activity and markedly higher lactate concentrations. Confirmatory studies from Experiment II found similar levels of elevated lactate and hypothermia, another sign consistent with mitochondrial impairment.

Energy sensor AMPK is amply expressed in brain and can be activated by energetic stress. Our experiments found high levels of AMPK activation in the Cu deficient cerebellum. Compared to controls, higher levels of pAMPK were detected in Cu⁻ cerebella at both ages of postnatal development analyzed in the current experiments. AMPK activation appears to be increasing in step with increasing CCO inhibition and lactate concentrations, suggesting AMPK dependent processes may be important in offsetting metabolic consequences of mitochondrial dysfunction.

When activated, one specific way AMPK helps maintain cellular energy stores is by shutting down biosynthetic processes like fatty acid synthesis by phosphorylating and inhibiting ACC, the rate limiting enzyme in this biosynthetic process (Brownsey et al., 2006; Munday, 2002). In the current experiments, at both ages in postnatal development, cerebellar levels of pACC were higher in Cu⁻ rat pups, consistent with impairment in enzyme activity. Cell culture studies suggest that AMPK is expressed in oligodendrocytes where ACC dependent and energetically costly myelin synthesis occurs (Moore and Brophy, 1994). Modulation of fatty acid biosynthesis during rapid brain growth is one way to spare the ATP pool during energetic deprivation stemming from mitochondrial inhibition. Our data suggest that AMPK activation and ACC inhibition may be one mechanism responsible for Cu deficiency induced delays in myelination and cerebellar synaptogenesis.

Studies of AMPK in various tissues have shown that its activation can stem from increased AMP concentrations occurring during energetic deprivation. However, it is clear from our data that differences in AMP cannot explain the augmentation in AMPK activity in Cu⁻ cerebella. Besides metabolic stressors leading to ATP depletion, there is a growing list of known stimuli leading to AMPK activation without apparent change in the AMP/ATP ratio (Altarejos et al., 2005; Fryer et al., 2002). Changes in AMP or other cellular factors can impact the upstream kinases and downstream phosphatases responsible for the phosphorylation state of AMPK

(Sanders et al., 2007). Covalent activation of AMPK occurs through phosphorylation of Thr 172 on its α subunit by AMPK kinases LKB1 or CaMMKK β (Ca²⁺/calmodulin-dependent protein kinase kinase β) (Sanders et al., 2007; Stein et al., 2000). CaMMKK β does not depend on AMP for action and may be one of the AMPK kinases potentially altered by Cu deficiency. Previous work in platelets, vascular endothelial cells, and cardiac myocytes all suggest that Ca²⁺/homeostasis is aberrant in Cu⁻ cells (Johnson and Dufault, 1993; Prohaska and Heller, 1999; Schuschke et al., 2000).

Another potential mediator of enhanced pAMPK is enhanced RNS (Zou et al., 2004). Others have previously reported enhanced 3-nitrotyrosine staining in neutral tube tissue of copper deficient embryos (Beckers-Trapp et al., 2006). Furthermore, cardiac tissue from Cu deficient rats was recently shown to have increased NOS activity and expression levels (Saari et al., 2007). Cu⁻ rat pups in the current experiments exhibited marked cardiac hypertrophy suggesting that severe Cu deficiency in cardiac tissue was evident (data not shown). However, despite our expectations, data from current experiments with perinatal Cu deficiency failed to detect alterations in plasma or cerebellar NO metabolites or in cerebellar 3-nitrotyrosine levels. Additional work will be required to identify the signal responsible for enhanced pAMPK content in Cu⁻ rat cerebella. Data in the current model suggest that neither elevated AMP or RNS are likely causes for enhanced pAMPK.

It is interesting to note that ATP levels remained constant in the Cu⁻ cerebellum. This has also been previously seen in the Cu deficient whole brain (Prohaska and Wells, 1975). The extent to which AMPK is involved in maintaining cerebellar ATP levels during Cu deficiency is a question for further exploration. Neuronal culture studies have shown that AMPK is important in cell survival in both cyanide induced mitochondrial inhibition and during glucose deprivation, suggesting that AMPK is important to neurons during energetic stress (Culmsee et al., 2001). Though in some tissues, like heart, AMPK helps maintain energetic homeostasis via modulation of glycolysis (Marsin et al., 2000), it is not yet clear if the same occurs in brain, though some studies suggest this mechanism occurs in astrocytes (Almeida et al., 2004). Interestingly, a combination of in vitro and in vivo studies have shown that by inhibiting ACC, AMPK activation in brain can drive ketone body production which can serve as an alternative, and preferred, fuel for neurons under conditions of mitochondrial inhibition (Blazquez et al., 1999). Further studies would be needed to assess the various roles AMPK activation and ACC inhibition can play in the Cu⁻ cerebellum and brain.

In conclusion, current experiments demonstrate that extensive mitochondrial inhibition in Cu⁻ cerebella is accompanied by AMPK activation and enhanced phosphorylation of the biosynthetic enzyme ACC, which may constitute a potential mechanism responsible for Cu deficiency induced inhibition of cerebellar biosynthetic processes.

4. Experimental procedures

4.1 Animal care and induction of Cu deficiency

Holtzman sperm-positive rats were purchased commercially (Harlan Sprague Dawley, Indianapolis, IN) and received either copper-adequate (Cu⁺) or copper-deficient (Cu⁻) dietary treatment consisting of a copper-deficient modified AIN-76A diet (Teklad Laboratories, Madison, WI) that contained 0.34 mg Cu/kg by analysis. Cu-supplemented, 20 mg Cu/L, or Cu⁻ deficient, deionized drinking water, were used to establish two treatment groups (Gybina and Prohaska, 2003). Offspring in this perinatal model of Cu deficiency were sampled at postnatal age 13 and 24 (P13 and P24) (Experiment I). Four dams of each treatment group and their litters were sampled. To confirm our results, a second set of Cu⁺ and Cu⁻ rats were generated as described above and sampled at P24 (Experiment II). Five dams of each treatment group were used. All animals were maintained at 24°C with 55% relative humidity on a 12-h

light cycle (0700–1900-h). All protocols were approved formally by the University of Minnesota Animal Care Committee.

Body temperature was determined in P24 pups in Experiment II by using a rectal probe in unanesthetized rats (Sensortek Thermalert TH8, Clifton, NJ).

4.2 Tissue collection

To prevent changes in metabolite concentrations induced by anesthetics, animals were decapitated without anesthesia. To minimize stress experienced by animals from handling before decapitation, rats were handled daily from P0 to day of tissue collection. Upon decapitation, cerebella were immediately removed from the skull and frozen in liquid nitrogen. Cerebellar extraction time did not differ between Cu⁺ and Cu[−] animals. There were 12 separate sampling functions including two ages and two experiments. The mean $\pm$ SEM time to freeze was 13.5 ± 0.5 seconds. Fast frozen cerebella were stored at -75°C until analysis. Remaining brain was collected for total Cu analysis. A separate cerebellum was used for metabolite analyses and for immunoblots. Thus, two littermate brothers were used for each time point from each original dam.

4.3 Biochemical analyses

Cerebellar CCO activity was determined spectrophotometrically in selected animals by protocols detailed elsewhere (Prohaska, 1983). The remainder of the brain was wet-digested with HNO_3 (Trace Metal grade; Fisher Scientific, Pittsburgh, PA) and samples were analyzed for Cu content by flame atomic absorption spectroscopy (Model 1100B, Perkin-Elmer, Norwalk, CT) (Prohaska, 1983). Protein content of tissue samples was determined using a modified version of the Lowry method (Markwell et al., 1978).

For measurement of cerebellar ATP, AMP, and lactate concentrations, cerebellar metabolite extracts were prepared and analyzed according to Lowry et al. with some modifications (Lowry and Passonneau, 1972). Briefly, fast frozen tissues were powdered under liquid nitrogen and then transferred to tubes, chilled on dry ice, and mixed with 5 volumes of 0.7M HClO_4 . Tubes were then transferred to a -8°C alcohol bath, where samples and acid were mixed. This mixture was then rapidly homogenized using a tissue probe cooled to 4°C . Homogenates were spun, supernatant neutralized with KHCO_3 and metabolite concentrations determined using enzymatic analyses as previously described (Lowry and Passonneau, 1972).

RNS were evaluated in two ways. First, NO metabolites, nitrite and nitrate, were analyzed following guidelines in the total nitric oxide assay kit (Assay Designs, Inc. Ann Arbor, MI). Briefly, nitrate is reduced to nitrite by nitrate reductase and the combined nitrite determined using the Griess reaction. We used duplicate $50\text{ }\mu\text{L}$ samples of plasma diluted with two volumes of buffer and $50\text{ }\mu\text{L}$ of cerebellar supernatant that was diluted with a total of 9 volumes of buffer after ultracentrifugation. All samples were ultrafiltered to remove most protein and the color was measured at 542 nm and compared to apropos standards. Some cerebellar samples required centrifugation after color development to remove precipitated protein. Second, we measured peroxynitrite production indirectly by detection of 3-nitrotyrosine cerebellar peptide abundance following immunoblot methods as described below.

4.4 Western blot analysis

Samples for western blot analysis were prepared by homogenizing fast frozen cerebella in 6 volumes of a $50\text{ mM Tris pH } 7.5$ buffer containing 50 mM NaF , $5\text{ mM sodium pyrophosphate}$, 250 mM sucrose , 1 mM EDTA , 0.5% Triton X-100, and protease inhibitors (Protease Inhibitor Cocktail, Sigma, St. Louis, MO). Homogenates were spun at $15,000 \times g$ for 15 minutes at 4°C . Supernatants were stored at -75°C until western blot analysis. Fractionation was carried

out by loading 40 µg of protein on SDS-PAGE gels, 10% gel for total AMPK and phospho-AMPK (pAMPK) and 6% gel for phosphorylated acetyl-CoA carboxylase (pACC) analysis. Proteins were transferred to 0.2 µm nitrocellulose membranes and processed for immunoblotting as described elsewhere (Prohaska and Brokate, 2001). Equal loading was verified by Ponceau S staining. Some membranes were reprobed after incubation of membranes with buffer containing 2-mercaptoethanol and SDS at 55°C.

Detection of total AMPK ($\alpha 1$ and $\alpha 2$) and pAMPK ($\alpha 1$ Thr¹⁷² and $\alpha 2$ Thr¹⁷²) was achieved using antibodies purchased from Cell Signaling Technology, Inc. and used at 1:1000 dilution, according to manufacturer instructions. pACC protein levels were determined using a phospho-ACC (Ser⁷⁹) specific antibody (1:1000, Upstate Biotechnology, Inc.). Copper chaperone for superoxide dismutase (CCS) detection was carried out as described elsewhere using a 1:1000 dilution of a previously characterized affinity purified antibody (West and Prohaska, 2004). 3-Nitrotyrosine levels were analyzed using an antibody and directions provided by a Nitrotyrosine Immunoblot Kit (Cell Biolabs, Inc.). A positive control, 3-nitrotyrosine labeled bovine albumin, was included in the immunoblot protocol; a 10% gel was used. Subunit IV of CCO, COX IV, protein levels were analyzed using mouse monoclonal anti-COX IV, at 1:4000 dilution (Molecular Probes, Eugene, OR) following fractionation on a 15% gel.

All membranes were blocked for an hour or more using 5% BSA/TBS containing 0.1% Triton X-100, and incubated overnight with primary antibodies at 4°C. All secondary species-specific antibodies were diluted 1:10,000. SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) was used for detection. Chemiluminescence was captured using high speed blue X-ray film (Lake Superior X Ray Inc., Duluth, MN) and densitometry was carried out using the FluorChem™ system (Alpha Innotech, San Leandro, CA).

4.5 Statistical analyses

Means $\pm$ SEM were calculated. Student's unpaired two-tailed *t*-test was used to compare data between the two dietary treatments, $\alpha = 0.05$ and $\alpha = 0.01$. Variance equality was evaluated using the F-test. All calculations were performed using Microsoft™ Excel.

Acknowledgments

We thank Margaret Boderius and Joshua Pyatskowitz for their technical assistance. Mark Walters assistance with the nitric oxide studies is appreciated. This work was supported by NIH HD-039708.

References

- Almeida A, Moncada S, Bolanos JP. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat. Cell. Biol* 2004;6:45–51. [PubMed: 14688792]
- Altarejos JY, Taniguchi M, Clanachan AS, Lopaschuk GD. Myocardial ischemia differentially regulates LKB1 and an alternate 5'-AMP-activated protein kinase kinase. *J. Biol. Chem* 2005;280:183–190. [PubMed: 15507450]
- Beckers-Trapp ME, Lanoue L, Keen CL, Rucker RB, Uriu-Adams JY. Abnormal development and increased 3-nitrotyrosine in copper-deficient mouse embryos. *Free Radic. Biol. Med* 2006;40:35–44. [PubMed: 16337877]
- Bertinato J, Iskandar M, L'Abbe MR. Copper deficiency induces the upregulation of the copper chaperone for Cu/Zn superoxide dismutase in weanling male rats. *J. Nutr* 2003;133:28–31. [PubMed: 12514262]
- Blazquez C, Woods A, de Ceballos ML, Carling D, Guzman M. The AMP-activated protein kinase is involved in the regulation of ketone body production by astrocytes. *J. Neurochem* 1999;73:1674–1682. [PubMed: 10501215]
- Brownsey RW, Boone AN, Elliott JE, Kulpa JE, Lee WM. Regulation of acetyl-CoA carboxylase. *Biochem. Soc. Trans* 2006;34:223–227. [PubMed: 16545081]

- Cohen E, Elvehjem CA. The relation of iron and copper to cytochrome and oxidase content of animal tissues. *J. Biol. Chem* 1934;107:97–105.
- Culmsee C, Monnig J, Kemp BE, Mattson MP. AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation. *J. Mol. Neurosci* 2001;17:45–58. [PubMed: 11665862]
- Danks DM, Campbell PE, Stevens BJ, Mayne V, Cartwright E. Menkes's kinky hair syndrome. An inherited defect in copper absorption with widespread effects. *Pediatrics* 1972;50:188–201. [PubMed: 5045349]
- El Meskini R, Crabtree KL, Cline LB, Mains RE, Eipper BA, Ronnett GV. ATP7A (Menkes protein) functions in axonal targeting and synaptogenesis. *Mol. Cell. Neurosci* 2007;34:409–421. [PubMed: 17215139]
- Everson GJ, Tsai HC, Wang TI. Copper deficiency in the guinea pig. *J. Nutr* 1967;93:533–540. [PubMed: 6082665]
- Fryer LG, Parbu-Patel A, Carling D. The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J. Biol. Chem* 2002;277:25226–25232. [PubMed: 11994296]
- Gybina AA, Prohaska JR. Increased rat brain cytochrome c correlates with degree of perinatal copper deficiency rather than apoptosis. *J. Nutr* 2003;133:3361–3368. [PubMed: 14608045]
- Gybina AA, Prohaska JR. Variable response of selected cuproproteins in rat choroid plexus and cerebellum following perinatal copper deficiency. *Genes and Nutrition* 2006;1:51–60.
- Johnson WT, Dufault SN. Intracellular calcium mobilization in rat platelets is adversely affected by copper deficiency. *Biochim. Biophys. Acta* 1993;1175:263–268. [PubMed: 8435442]
- Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell. Metab* 2005;1:15–25. [PubMed: 16054041]
- Lowry, OH.; Passonneau, JV. A flexible system of enzymatic analysis. New York: Academic Press; 1972.
- Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem* 1978;87:206–210. [PubMed: 98070]
- Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr. Biol* 2000;10:1247–1255. [PubMed: 11069105]
- Menkes JH, Alter M, Steigleder GK, Weakley DR, Sung JH. A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. *Pediatrics* 1962;29:764–779. [PubMed: 14472668]
- Mercer JF. Menkes syndrome and animal models. *Am. J. Clin. Nutr* 1998;67:1022S–1028S. [PubMed: 9587146]
- Moore F, Brophy PJ. Regulation of acetyl-CoA carboxylase (ACC) by ATP depletion in developing oligodendrocytes mimics the action of AMP-activated protein kinase (AMPK). *Biochem. Soc. Trans* 1994;22:416S. [PubMed: 7698436]
- Munday MR. Regulation of mammalian acetyl-CoA carboxylase. *Biochem. Soc. Trans* 2002;30:1059–1064. [PubMed: 12440972]
- Penland JG, Prohaska JR. Abnormal motor function persists following recovery from perinatal copper deficiency in rats. *J. Nutr* 2004;134:1984–1988. [PubMed: 15284387]
- Prohaska JR, Wells WW. Copper deficiency in the developing rat brain: a possible model for Menkes' steely-hair disease. *J. Neurochem* 1974;23:91–98. [PubMed: 4368537]
- Prohaska JR, Wells WW. Copper deficiency in the developing rat brain: evidence for abnormal mitochondria. *J. Neurochem* 1975;25:221–228. [PubMed: 169321]
- Prohaska, JR. Changes in Brain Enzymes Accompanying Deficiencies of the Trace Elements, Copper, Selenium, or Zinc. In: Howell, JMcC; Gawthorne, JM.; White, CL., editors. *Trace Element Metabolism in Man and Animals (TEMA-4)*. Canberra: Australian Academy of Science; 1981. p. 275-282.

- Prohaska JR. Changes in tissue growth, concentrations of copper, iron, cytochrome oxidase and superoxide dismutase subsequent to dietary or genetic copper deficiency in mice. *J. Nutr* 1983;113:2048–2058. [PubMed: 6312000]
- Prohaska JR, Hoffman RG. Auditory startle response is diminished in rats after recovery from perinatal copper deficiency. *J. Nutr* 1996;126:618–627. [PubMed: 8598546]
- Prohaska JR, Heller LJ. Calcium reintroduction decreases viability of cardiac myocytes from copper-deficient rats. *J. Nutr* 1999;129:1842–1845. [PubMed: 10498756]
- Prohaska JR, Brokate B. Dietary copper deficiency alters protein levels of rat dopamine beta-monooxygenase and tyrosine monooxygenase. *Exp. Biol. Med* 2001;226:199–207.
- Prohaska JR, Broderius M, Brokate B. Metallochaperone for Cu,Zn-superoxide dismutase (CCS) protein but not mRNA is higher in organs from copper-deficient mice and rats. *Arch. Biochem. Biophys* 2003;417:227–234. [PubMed: 12941305]
- Saari JT, Wold LE, Duan J, Ren J, Carlson HL, Bode AM, Lentsch AB, Zeng H, Schuschke DA. Cardiac nitric oxide synthases are elevated in dietary copper deficiency. *J. Nutr. Biochem* 2007;18:443–448. [PubMed: 16997540]
- Sanders MJ, Grondin PO, Hegarty BD, Snowden MA, Carling D. Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem. J* 2007;403:139–148. [PubMed: 17147517]
- Schuschke DA, Falcone JC, Saari JT, Fleming JT, Percival SS, Young SA, Pass JM, Miller FN. Endothelial cell calcium mobilization to acetylcholine is attenuated in copper-deficient rats. *Endothelium* 2000;7:83–92. [PubMed: 10865936]
- Shoubridge EA. Cytochrome c oxidase deficiency. *Am. J. Med. Genet* 2001;106:46–52. [PubMed: 11579424]
- Spencer EB, Bianchi A, Widmer J, Witters LA. Brain acetyl-CoA carboxylase: isozymic identification and studies of its regulation during development and altered nutrition. *Biochem. Biophys. Res. Commun* 1993;192:820–825. [PubMed: 8097913]
- Stein SC, Woods A, Jones NA, Davison MD, Carling D. The regulation of AMP-activated protein kinase by phosphorylation. *Biochem. J* 2000;345(Pt 3):437–443. [PubMed: 10642499]
- West EC, Prohaska JR. Cu,Zn-superoxide dismutase is lower and copper chaperone CCS is higher in erythrocytes of copper-deficient rats and mice. *Exp. Biol. Med* 2004;229:756–764.
- Zimmerman AW, Matthieu JM, Quarles RH, Brady RO, Hsu JM. Hypomyelination in copper-deficient rats. Prenatal and postnatal copper replacement. *Arch. Neurol* 1976;33:111–119. [PubMed: 1252145]
- Zou MH, Kirkpatrick SS, Davis BJ, Nelson JS, Wiles WGt, Schlattner U, Neumann D, Brownlee M, Freeman MB, Goldman MH. Activation of the AMP-activated protein kinase by the anti-diabetic drug metformin in vivo. Role of mitochondrial reactive nitrogen species. *J. Biol. Chem* 2004;279:43940–43951. [PubMed: 15265871]

Abbreviations used

AMPK, AMP-activated protein kinase
 pAMPK, phosphorylated AMPK
 ACC, acetylCoA carboxylase
 pACC, phosphorylated ACC
 CCS, copper chaperone for superoxide dismutase
 CCO, cytochrome c oxidase
 COX IV, subunit IV of cytochrome c oxidase
 Cu, copper
 Cu+, copper adequate
 Cu–, copper deficient
 P13, postnatal day 13
 P24, postnatal day 24
 RNS, reactive nitrogen species

Fig. 1. Copper deficiency induces mitochondrial dysfunction

In Experiment I (panel A), cerebellar Cu deficiency was associated with reduced cytochrome c oxidase activity (CCO) as measured in Cu⁻ P13 and P24 animals and compared to age matched controls (n=4). Mitochondrial dysfunction was further evidenced by higher cerebellar lactate concentration in Cu⁻ P13 and P24 animals compared to age-matched controls (n=4). In a confirmatory Experiment II (panel B), body temperature of Cu⁻ P24 rats was significantly lower compared to Cu⁺ controls, suggesting mitochondrial inhibition (n=5). Cerebellar lactate of Cu⁻ P24 animals was markedly higher than Cu⁺ controls (n=5). Values are mean $\pm$ SEM; * indicates $P < 0.01$.

Fig. 2. Copper deficiency activates cerebellar AMPK throughout postnatal development
 Cu- cerebella (-) have higher levels of phosphorylated AMPK (pAMPK) than Cu+ controls (+) at both P13 (panel A) and P24 (panel B). Antibody specifically detected pAMPK ($\alpha 1$ Thr¹⁷² and $\alpha 2$ Thr¹⁷²). Total AMPK, both $\alpha 1$ and $\alpha 2$, was unchanged by Cu deficiency, though cerebella were indeed Cu deficient as shown by higher CCS protein levels in Cu- than Cu+ samples. Equal loading of 40 μ g protein per lane was visualized by Ponceau S staining of the membrane (data not shown). Normalized densities were obtained by dividing individual pAMPK band densities by the average Cu+ control density. Bar graph values are means $\pm$ SEM; * indicates $P < 0.01$; ** indicates $P < 0.05$.

Fig. 3. Increased phosphorylation of ACC detected in Cu-cerebella

To confirm the physiological relevancy of AMPK activation by Cu deficiency, phosphorylation of serine 79 in acetyl CoA carboxylase (ACC), a specific target for activated AMPK, was analyzed in P13 and P24 cerebella (Cb) using immunoblots. At both ages, levels of pACC were statistically higher in Cu- cerebella compared to Cu+ controls consistent with functional AMPK activation by Cu deficiency. Normalized densities were obtained by dividing all individual pACC band densities by the average Cu+ control density. Equal loading of protein was visualized with Ponceau S stain. Bar graph values are means $\pm$ SEM; * indicates $P < 0.01$; ** indicates $P < 0.05$.

Fig. 4. Reactive nitrogen species are not elevated in Cu- cerebella

Cu+ and Cu- rat pups were analyzed for reactive nitrogen species (RNS) by performing western blots of cerebellar homogenates, 50 µg protein, for 3-nitrotyrosine (Panel A). One 3-nitrotyrosine positive band, 30 kDa, was detected using a 3-nitrotyrosine antibody. There were no statistical differences between 3-nitrotyrosine levels in Cu+ and Cu- cerebella extracts. Western blots were also probed for CCS, a marker of Cu deficiency, which was higher in Cu- cerebella compared to Cu+. Equal loading of samples was confirmed by probing for actin. The same cerebellar samples were analyzed on a separate western blot (25 µg/lane) for COX IV content to establish mitochondrial inhibition. COX IV abundance was strikingly lower in Cu- samples compared to Cu+. RNS levels were also analyzed by determining NO metabolite (nitrite plus nitrate) levels in Cu+ and Cu- cerebellar extracts (Panel B; n=4). No significant differences between Cu+ and Cu- cerebellar NO metabolite concentrations were detected, $P > 0.05$.

Table 1

ATP and AMP concentrations in copper-adequate (Cu+) and copper-deficient (Cu-) male rat cerebella following perinatal copper deficiency.

Metabolite	Experiment I		Experiment II	
	P13 Cu+	Cu-	P24 Cu+	Cu-
ATP, $\mu\text{mol/g}$	1.45 ± 0.03	1.41 ± 0.03	1.47 ± 0.03	1.50 ± 0.03
AMP, $\mu\text{mol/g}$	0.20 ± 0.01	0.21 ± 0.02	0.23 ± 0.03	0.19 ± 0.01
AMP/ATP	0.14 ± 0.01	0.15 ± 0.02	0.16 ± 0.02	0.13 ± 0.01

Metabolites were measured spectrophotometrically using enzyme coupled assays (Lowry and Passonneau, 1972). Values are means $\pm$ SEM (n=4 or 5). No significant treatment differences were detected, $P > 0.05$.