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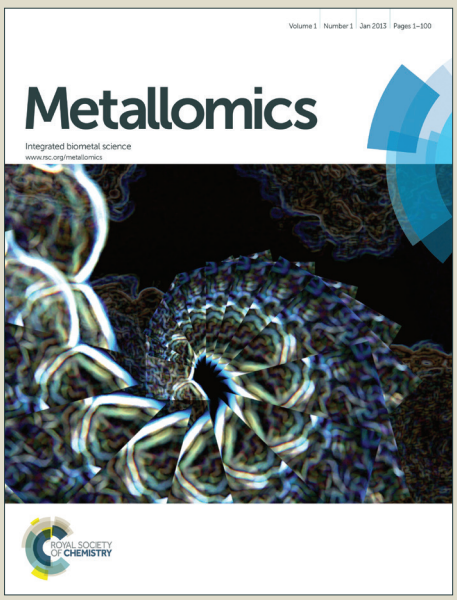

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Hepatic metabolic response to restricted copper intake in a Niemann-Pick C murine model.

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Niemann–Pick C disease (NPC) is a vesicular trafficking disorder primarily caused by mutations in the *Npc1* gene and characterized by liver dysfunction and neuropathology. Altered hepatic copper metabolism has recently been reported in NPC disease. Therefore, we aimed to analyze the effects of a copper deficient diet and copper chelation using D-penicillamine on copper homeostasis in the liver of *Npc1*$^{-/-}$ mice at different ages. We examined liver metal ions content by AAS, and copper and iron metabolism gene expression in the liver using qPCR in *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. We found higher copper and lower iron content in the liver of *Npc1*$^{-/-}$ mice of different ages, compared to controls; these changes in copper and iron content were correlated with increased *ceruloplasmin*, *metallothionein 1*, *transferrin receptor* gene expression and decreased gene expression of *Commd1*, *ferritin-light chain* and *ferroportin* in the livers of *Npc1*$^{-/-}$ mice of different ages. *Npc1*$^{-/-}$ mice responded to a copper-deficient diet with a decrease in copper content in the liver, bile and heart. These results correlated with a reduction in the hepatic expression of *ceruloplasmine* and *metallothionein 1* during the first weeks of treatment. D-penicillamine revealed hepatic adaptive response and an improvement in hepatic function in *Npc1*$^{-/-}$ mice without effects on neurological function. Our results confirm that the NPC1 protein is required for copper and iron homeostasis. To our knowledge, this is the first report documenting the hepatic adaptive response to low-copper intake in a *Npc1*$^{-/-}$ mice model.

Introduction

Niemann-Pick type C (NPC) disease is a rare lysosomal disorder caused by the genetic loss of NPC1 or NPC2 function, characterized by liver dysfunction and progressive neurodegeneration[1,2]. Approximately 95% percent of NPC patients have mutations in the *Npc1* gene, and 5% have mutations in the *Npc2* gene[3]. At the cellular level, mutations in the *Npc1* gene result in multiple vesicular trafficking defects and accumulation of lysosomal material, mainly cholesterol, that are potentially deleterious to health[4,5]. Thus, a deficiency in this protein alters intracellular lipid homeostasis, membrane properties and intracellular trafficking of organelles[6]. Although neurodegeneration is a major feature of NPC, many patients present neonatally with acute liver disease that can be fatal but if survived spontaneously resolves[1,7].

Iron and copper are essential dietary components required to meet the demands from cell growth, differentiation, and optimal homeostasis[8,9]. However, both are redox active metals and through the Fenton reaction, both cuprous and ferrous ion transform weak oxidant hydrogen peroxide into hydroxyl radicals, one of the most reactive species in nature[10,11]. Iron and copper depend, at least in part, on vesicular trafficking for their cellular uptake[12] and efflux[13], respectively. In hepatocytes, the principal uptake pathway of iron bound to transferrin (TF) is through endocytosis mediated by the transferrin receptor (TFR)[12]. Once the iron-TF/TFR complex is internalized via endocytosis, it is delivered to the early/sorting endosome through vesicular trafficking[14]. Interestingly, inefficient recycling of TFR has been reported in NPC cells[15]. After release from TF, iron is transported across endosomal membranes into the transit pool within the cytosol, where it regulates ferritin (*Ft*) translation[16]. Previous studies in liver tissues from NPC patients described FTH and FTL scarcity and suggested that injuries in NPC1 block the intracellular utilization not only of cholesterol but also of iron, impairing the synthesis of cytosolic FT[17]. Also, recently Hung et al. reported altered iron and copper homeostasis in a murine model and in NPC patients[18] and another study described serum free copper with low ceruloplasmin (CP) in a NPC patient[19]. Therefore, abnormal iron and copper metabolism may be components of the pathogenic cascade in NPC disease. At the cellular level, once copper is in the cytoplasm of hepatocytes, it can be transferred to ATP7B through copper chaperones such as ATOX1[20,21]. The primary role of ATP7B is to transport copper from the cytosol into the secretory compartment of the cell, where copper can be incorporated into newly synthesized cuproproteins or distributed through vesicles to the lysosomal pathway, or to the plasma membrane for copper export, while the ATP7B protein itself is recycled via the trans-Golgi network (TGN)[13, 22,23]. When cellular copper increases, ATP7B undergoes intracellular redistribution and promotes excretion of excess copper into bile[13, 24,25]. Recently, reports indicated that copper excretion may be impaired in NPC hepatic cells due to the disruption of the late

endosome to TGN transport[26] and that liver copper content increase in *in vivo* models of NPC disease[27].

Here, we investigated the role of NPC1 in iron and copper metabolism by analyzing copper/iron liver storage, copper excretion and copper/iron metabolism-related gene expression. We compared *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice at 4, 7, and 8-week-old, this age range of mice represents an early to intermediate time period before the premature death of *Npc1*$^{-/-}$ mice on the BALB/c background that typically occurs at the age of 10-12 weeks[28,29]. We found higher copper and lower iron content in the liver of *Npc1*$^{-/-}$ mice with respect to controls (*Npc1*$^{+/+}$) at all ages; copper and iron content correlated with changes in copper and iron gene expression pattern in the liver of four- and seven-week-old *Npc1*$^{-/-}$ mice. Considering that excess liver copper has recently been reported in NPC mice model and associated with hepatic damage[27,30], we analyzed the effects of a copper-deficient diet and effects of copper chelator D-penicillamine (DPA) in copper homeostasis in *Npc1*$^{-/-}$ mice at different ages. We examined the copper content of the liver, bile, plasma and heart by AAS, and copper metabolism gene expression in the liver by qPCR. *Npc1*$^{-/-}$ mice responded to a copper-deficient diet with a decrease in copper content in the liver, bile and heart. These results were correlated with a reduction in the hepatic expression of *Cp* and *metallothionein 1 (Mt1)* in the first weeks of treatment. DPA reduced the transaminases levels in eight-week-old *Npc1*$^{-/-}$ mice. Our results suggest that NPC1 function is relevant for copper and iron homeostasis. To our knowledge, this is the first report documenting the hepatic adaptive response to low copper intake in a NPC mice model.

Materials and methods

Animals

Niemann-Pick type C1 mice (BALB/c Nctr-Npc1^{m1N}/J, *Npc1*$^{-/-}$ mice) carrying a mutation in the *Npc1* gene[31] were from an established colony. Genotypes were identified using PCR-based screening, as described previously[32]. Immediately after weaning, Balb/c male mice were fed ad libitum for 4 weeks with a copper adequate (Cu-A) or copper-deficient (Cu-D) AIN-76A rodent diet (Research Diets, Inc.,New Brunswick, NJ, catalogue D18106 and D18104, respectively) containing 6 mg and 0.3 mg Cu/kg, respectively. All mice had free access to double-distilled water[33]. Mice of 4, 5, 7 or 8 weeks of age were fasted for 2 hours and then anesthetized by intraperitoneal injection of ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg) for tissue sampling. Animal studies performed in Departamento de Gastroenterología, Facultad de Medicina, Pontificia Universidad Católica de Chile were conducted using protocols defined by the Public Health Service Policy on Human Care and Use of Laboratory Animals in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals[34] and were approved by the review board for animal studies at our institution (Comité de Ética y Bienestar Animal, CEBAMedUC; Approval ID#005-2011). Animal studies performed in Department of Pharmacology, University of Oxford were conducted using protocols approved by the UK Home Office for the conduct of regulated procedures under license (Animal scientific Procedures Act, 1986).

Quantification of iron and copper

Liver and heart metal content was quantified as previously described[35]. Briefly, iron and copper were measured using a graphite furnace AAS (Perkin Elmer, SIMMA 6100). Iron and copper contents in plasma and bile were measured using a graphite furnace AAS (Perkin Elmer, SIMMA 6100) without pretreatment with nitric acid.

Echocardiography

Transthoracic M-mode of left ventricular was obtained via echocardiogram equipped with an 8 MHz transducer (ATL 5000 ultrasound machine), using a method previously described[36]. The cavity sizes of left ventricular end-diastolic and end-systolic internal dimensions (LVIDd and LVIDs) and left ventricular anterior (LVWT) and posterior wall or interventricular septum (IVS) thickness were measured. The heart rate was monitored and the left ventricular fractional shortening and the left ventricular ejection fraction were calculated using a modification of the American Society of Echocardiography method[37]. All echocardiography were performed and interpreted by a single operator blinded to the experiment.

RNA extraction

Total RNA was extracted from homogenized liver with TRI Reagent (Ambion, Carlsbad, CA, USA) according to manufacturer instructions. RNA quality and quantity were assessed prior to and after DNase digestion by denaturing gel electrophoresis and photometric analysis (A260/280 ratio), respectively.

cDNA synthesis and quantitative real-time quantitative PCR (qPCR)

Total RNA (2 μg) was used as a template for reverse transcription to synthesize single-stranded cDNA. Real-time quantitative PCR (qPCR) was performed as previously described[27]. Gene-specific primer sets detailed in Table S1, were designed using Primer3Plus to amplify DNA products between 70 and 200 bp. The following standard thermal profile was used: 10 min at 95°C, 40 cycles of 10 s at 95°C and 15 s at 60°C, with a final 10 s stage at 72°C. Data were analyzed using LightCycler Software (v.3, Roche). Efficiency was determined for each sample and each gene by LinRegPCR v.7.5 using data obtained from the exponential phase of each amplification plot[38]. The products were resolved by 2% agarose gel electrophoresis to confirm the presence of DNA fragments of the expected sizes. Transcript levels of genes were normalized to the *Ppia* gene[27], which was validated in our experimental conditions. qPCR was performed in samples from at least five mice.

D-penicillamine treatment

Npc1$^{-/-}$ mice treated with the copper chelating reagent, using DPA (100 mg/kg/day, Sigma)[39], supplemented as dry admix to powdered RM1 mouse chow (SDS, UK) or dissolved in drinking water (as indicated in legends of figure 4-7). These therapies were administered from 3 or 4 weeks of age to evaluate neurological and hepatic effects, respectively.

Locomotor Activity: AmLogger and Open Field Rearing

Spontaneous activity of each mouse was recorded weekly using both an automated activity monitor (AmLogger, Linton Instrument, UK) as described previously[40].

Gait Analysis

The CatWalk™ system (Noldus Information Technology, the Netherlands) was used for monitoring changes in movement patters as indicative of any motor complications and to assess any gait improvement in response to treatment[41]. *Npc1*$^{-/-}$ mice were subjected to CatWalk™ analysis at 9 weeks of age, where a minimum of three "runs" were collected per animal. The data collected were subsequently examined by CatWalk™ XT 10.0 software to produce of 177 inter –paw and intra-paw comparison parameters.

Serum Aminotransferases

Serum were collected from DPA in drinking water treated mice and then alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by the ALAT or the ASAT kit respectively (Kovalent, Rio de Janeiro, Brazil) following manufacturer's instructions.

Statistical analysis

GraphPad Prism v5 software was used for the statistical analysis. Mann–Whitney U test and unpaired *t* test, as indicated in figures legends were performed. Data are presented as mean±SEM as indicated. Statistical significance was defined as p □ *0.05*.

Results

Altered copper and iron homeostasis in the *Npc1*$^{-/-}$ mouse model

First, we used the AAS method to determine the total contents of copper and iron in liver and plasma, and bile copper levels, showed in Fig. 1A-E; from 4, 7, and 8-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. Fig. 1A shows a higher copper content and Fig. 1B shows a lower iron content in the liver of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice at 4, 7, and 8 weeks of age (p □ *0.05*). In addition, we determined the copper and iron contents in plasma in 4, 7, and 8-week-old mice as a representation of the availability of these two metals at the whole-body level, as shown in Fig. 1C-D. The same liver pattern was observed in plasma: an increase in copper and a decrease in iron were detected in 4, 7, and 8-week-old *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice. Plasma copper was 55 and 49 and 155% higher at 4, 7, and 8-week-old *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice. Plasma iron was 19 and 30 and 31% lower at 4 and 7 and 8-week-old *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice. These inverse correlations between intracellular copper and iron content in hepatic tissue and plasma agrees with recent studies by Hung et al., also in the *Npc1*$^{-/-}$ mouse model[18]. Considering that biliary secretion is the most important excretory mechanism to eliminate hepatic copper overload[42], we also quantified the copper content in bile, as shown in Fig. 1E. A marked decrease of copper content in bile of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice was observed, suggesting that accumulation of copper was due to a deficiency in hepatic copper excretion in the mutant mice. Taken together, these results indicate that copper and iron homeostasis are altered by the absence of NCP1 irrespective of age (4 or 7 or 8-week-old mice) and whether pre- or post-weaning. These results suggest that copper and iron metabolism alterations in *Npc1*$^{-/-}$ mice have an early onset, during intrauterine growth or post-natal prior to weaning.

Alterations in hepatic copper and iron metabolism-related gene expression in *Npc1*$^{-/-}$ mice

Cellular adaptation to high or low levels of metals depends on metabolic regulation mechanisms, which control uptake, intracellular handling, storage and efflux, usually by the functions of specific proteins, many of which are transcriptionally regulated[43]. To better understand the molecular mechanisms responsible for copper and iron alterations in livers of *Npc1*$^{-/-}$ mice, we quantified the mRNA abundance of copper metabolism genes, as shown in Fig. 1F and Table S2, and iron metabolism–related genes, as shown in Fig. 1G and Table S3, in the livers of 4- and 7-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice using qPCR. We choose 4- and 7-week-old to analyze the copper and iron gene expression patron, because until 7-week-old, *Npc1*$^{-/-}$ mice are in a steady state with respect to food intake and weight gain but not later, as described by Xie et al.[44]

We observed in Fig. 1F, the same copper metabolism-related gene expression pattern in 4- and 7-week-old mice. The expression of the copper storage gene *Mt1* and the ferroxidase enzyme *Cp* increased, whereas the expression of the intracellular copper handling gene *Commd1* decreased significantly in the liver of $Npc1^{-/-}$ mice compared to $Npc1^{+/+}$ mice ($p < 0.05$). We observed in Fig. 1D, that *Ctr1, Atox1,* and *Atp7b* gene expression showed no differences between genotypes. Regarding iron metabolism-related genes, we found a significant increase in the expression of the cellular iron uptake gene *TfR* and of the master regulator of iron metabolism gene *Hepc,* and a significant decrease in the expression of the iron storage and efflux genes *FtL* and *Fpn,* respectively, at 4 week-old $Npc1^{-/-}$ liver mice compared with $Npc1^{+/+}$ mice ($p < 0.05$). We observed in Fig. 1G that *Dmt1* and *FtH* gene expression showed no differences between genotypes. At 7 weeks, Fig. 1G shows a significant decrease of *Hepc* mRNA abundance in $Npc1^{-/-}$ compared to $Npc1^{+/+}$ mice ($p < 0.05$).

Effect of a copper-deficient diet on copper hepatic metabolism in the *Npc1*$^{-/-}$ mouse model

Since excess copper in the tissue can create favorable conditions for redox stress and oxidative tissue damage, we treated 4-week-old $Npc1^{-/-}$ and $Npc1^{+/+}$ mice with a copper-deficient diet for one or four weeks to help reduce liver and plasma copper levels. Fig. 2A shows a significant decrease of copper content in the liver of $Npc1^{-/-}$ mice at 5 and 8 weeks of age compared to 4-week-old $Npc1^{-/-}$ mice ($p < 0.05$). At 8 weeks the copper content of $Npc1^{-/-}$ mice reached values close to those of $Npc1^{+/+}$ mice, including $Npc1^{+/+}$ mice treated for one week with the copper-deficient diet (8.0±0.3 and 7.8±0.7 μg Cu/g dry weight, respectively). Also, the Fig. 2A shows a significant decrease of copper content in the liver of $Npc1^{-/-}$ mice at 5 weeks of age who were fed a copper-deficient diet compared to $Npc1^{-/-}$ mice fed a control diet ($p < 0.05$). Additionally, the Fig. 2A shows a significant decrease of copper content in the liver of $Npc1^{-/-}$ mice at 8 weeks of age who were fed a copper-deficient diet compared to $Npc1^{-/-}$ mice fed a control diet ($p < 0.05$). The liver copper content decreased during the first week of treatment in both $Npc1^{+/+}$ and $Npc1^{-/-}$ mice, but the decrease was significantly greater in $Npc1^{-/-}$ mice. As shown in Fig. 2B, no differences were observed in the $Npc1^{-/-}$ mice plasmatic copper levels after the first week of treatment. However, we observed in Fig. 2B, a significant decrease of copper content in the plasma of 8-week-old $Npc1^{-/-}$ mice compared to $Npc1^{-/-}$ mice fed with a control diet ($p < 0.05$). $Npc1^{+/+}$ mice showed no differences in the plasma copper level between treatments at any age, as shown in Fig. 2B. We also quantified copper in bile. Fig. 2C shows a small but significant decrease in biliary copper content was found in 5- and 8-week-old $Npc1^{-/-}$ mice fed a copper-deficient diet compared to $Npc1^{-/-}$ mice fed a control diet ($p < 0.05$). We also observed in Fig. 2C, a decrease in the biliary copper content of 5- and 8-week-old $Npc1^{+/+}$ mice fed a copper-deficient diet compared to $Npc1^{+/+}$ mice fed a control diet ($p < 0.05$).

Finally, we analyzed the effect of a copper-restricted diet in the body weight of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice and on the life span of *Npc1*$^{-/-}$ mice. The copper-restricted diet no altered the body weight of *Npc1*$^{+/+}$ mice or from *Npc1*$^{-/-}$ mice, Fig. S1A. Fig. S1B shows that *Npc1*$^{-/-}$ mice fed with a CuA diet lived an average of 9.8 weeks and when *Npc1*$^{-/-}$ mice fed with a low-copper diet, they lived an average of 10.4 weeks, as has been previously described by Alvarez et al.[45] and Maue et al.[29]

To understand the molecular mechanisms responsible for hepatic adaptive response in liver of *Npc1*$^{-/-}$ mice fed a copper-deficient diet for one week, we measured the expression of genes related with the uptake, intracellular handling, storage and efflux of copper in the livers of 5-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice fed control and copper-deficient diets using qPCR. As shown in Fig. 2D and Table S4, the *Npc1*$^{-/-}$ mice fed a copper-deficient diet decreased the expression of the ferroxidase enzyme gene *Cp* and the copper storage gene *Mt1* compared to *Npc1*$^{-/-}$ mice fed a control diet ($p < 0.05$). We observed in Fig. 2D that the *Ctr1, Atox1, Atp7b* and *Commd1* gene expression showed no differences between treatments. The *Npc1*$^{+/+}$ mice fed copper-deficient diets for one week showed no copper-related gene expression differences between treatments, as shown in Fig. 2D. These results indicate that hepatic adaptive response to low intake of copper in the *Npc1*$^{-/-}$ mice model was correlated with the changes of copper-related gene expression patterns in the liver.

Effect of a copper-deficient diet on heart copper content, in the cardiac morphology and cardiac function in the *Npc1*$^{-/-}$ mouse model.

To study the effect of a copper-deficient diet in extra hepatic copper metabolism in 5-and 8-week-old *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice we determined copper content in heart tissue. We found a significant decrease of copper content in the heart of *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice in the first week of intake of a copper-deficient diet compared with controls, *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice fed a control diet ($p < 0.05$). In the Fig. 3A we observed that at 8 weeks of age *Npc1*$^{-/-}$ and *Npc1*$^{+/+}$ mice showed no differences between treatments. Previous studies showed that mice fed a copper-deficient diet for 4 weeks, changed their cardiac phenotypes, increasing the thickness of the left ventricular and the interventricular walls[46]. We also analyzed the cardiac phenotype and function by echocardiography mode-M. We found a significant decrease of the thickness left ventricular and the interventricular walls at 3 weeks in *Npc1*$^{-/-}$ compared with *Npc1*$^{+/+}$ mice fed control diets ($p < 0.05$), as shown in Fig. 3B-D. In the Fig. 3C we observed that the left ventricular internal dimension showed no differences between genotypes. Analysis of the heart rate, the left ventricular fractional shortening and the ejection fraction indicated that cardiac function was not affected, as shown in Table S5. We also found a significant decrease in the thickness of the left ventricular and the interventricular walls in 4-week-old *Npc1*$^{-/-}$ mice fed a copper-deficient diet for one week compared with 4-week-old *Npc1*$^{-/-}$ mice fed for one week with a copper-adequate diet ($p < 0.05$), as shown in Fig. 3E-G. In

the Fig. 3F we observed that the left ventricular internal dimension showed no differences between treatments. At 5 weeks *Npc1*$^{+/+}$ mice showed no differences between treatments, as shown in Fig. 3E-G. Analysis of the heart rate, the left ventricular fractional shortening and the ejection fraction indicated that cardiac function was not affected between genotypes, as shown in Table S2. Together these results imply that *Npc1*$^{-/-}$ mice responded to a decrease in the dietary supply of copper by modulating both their cardiac copper content and cardiac phenotypes changes.

Effect of D-penicillamine copper chelator on copper levels in the liver, bile and plasma, and liver function of *Npc1*$^{-/-}$ mouse model.

In order to study the effect of a therapeutic intervention for NPC patients we treated *Npc1*$^{-/-}$ mice with DPA, an effective copper chelator used for treatment of Wilson disease[47,48]. DPA was administrated in drinking water (100 mg/kg weight of mice/day) from 4 until 8 weeks of age[39]. The results showed a significant decrease of copper content in the bile of *Npc1*$^{-/-}$ mice treated with DPA compared to *Npc1*$^{-/-}$ mice that received drinking water ($p \leq 0.05$). In the Fig. 4B we observed that the bile of *Npc1*$^{+/+}$ mice showed no copper content differences between DPA or drinking water treatments. Liver and plasma copper content showed no differences between DPA or drinking water treatment between genotypes, as shown in Fig. 4A-C. In Fig. 5A-B we observed a significant decrease of AST and ALT plasma activity in DPA treated *Npc1*$^{-/-}$ mice compared to *Npc1*$^{-/-}$ mice that received drinking water ($p \leq 0.05$). As illustrated in Fig. 5A-B, the AST and ALT plasma activity showed no differences between *Npc1*$^{+/+}$ treated with DPA or drinking water. These results indicate that although DPA does not change total copper content in liver and plasma it is capable of improving liver function in *Npc1*$^{-/-}$ mice.

Effect of D-penicillamine copper chelator on neurological function of *Npc1*$^{-/-}$ mouse model.

Next, to evaluate the potential therapeutic effects of DPA another group of mice were treated from 3 to 9 weeks of age with DPA (100 mg/kg/day) supplemented as dry admix and neurological functions of *Npc1*$^{-/-}$ were evaluated. Locomotor activity DPA treated *Npc1*$^{-/-}$ mice were measured by AmLogger measurements[40]. There was no significant improvement in locomotors activity, including FR count, activity (S), mobile (S), rearing (S), activity and rearing counts, in DPA-treated *Npc1*$^{-/-}$ mice while compared with normal powder diet treated *Npc1*$^{-/-}$ mice, as shown in Fig. S2A-F. The effects of DPA on gait were also measured in 9-week-old *Npc1*$^{-/-}$ mice[41]. As illustrated in Fig. 6A-F, no significant improvements on gait were found in DPA treated 9-week-old *Npc1*$^{-/-}$ mice. Therefore, these results suggested DPA, has limited therapeutic effect on neurological functions in treated *Npc1*$^{-/-}$ mice.

Discussion

The results of our study indicate that *Npc1*$^{-/-}$ mice, which exhibit signs of copper overload and iron depletion in liver and plasma immediately after weaning, responded rapidly to a copper-deficient diet reducing copper content in the liver, bile and heart. These responses were correlated with liver and heart changes in the copper-related gene expression profile and the cardiac phenotype, respectively. These results suggest that *Npc1*$^{-/-}$ mice can sense copper deficiency early in life. In addition, *Npc1*$^{-/-}$ mice responded to a copper chelator DPA treatment for four weeks, reducing biliary copper content and plasma transaminases, ALT and AST, levels. These results indicate that although DPA does not change total copper content in the liver and plasma, it does improve liver function in *Npc1*$^{-/-}$ mice. To our knowledge, this is the first report documenting the hepatic adaptive response to low copper intake, obtained through dietary intervention or by using a copper-chelating compound, in NPC mouse models.

Our data indicate that hepatic copper and iron metabolism alterations in *Npc1*$^{-/-}$ mice have an early onset that is maintained over time, even before weight loss and major neurological symptoms, which occur after 7 weeks of age[3, 44,28]. In this context, further analyses are needed to determine whether these early changes in copper and iron hepatic metabolism are also present in models with more slowly progressing forms of NPC disease, e.g., *Npc1*^{nmf164} mice[29]. Similar results were recently reported in the liver and plasma of the *Npc1*$^{-/-}$ mouse model by Hung et al.[18] extending our previous findings of altered hepatic copper metabolism using *Npc1*$^{-/-}$ mice[27]. Moreover, Hung et al.[18] reported disrupted metal homeostasis in human patient cerebrospinal fluid, plasma and post-mortem brain tissues and another study described serum-free copper with low CP in a NPC patient[19]. Therefore, abnormal copper and iron metabolism may be part of the alterations involved in NPC disease.

Characterization of the copper and iron metabolism in NPC mice model at 4 and 7 weeks of age.

In order to understand the molecular mechanisms associated with alterations of hepatic copper metabolism in *Npc1*$^{-/-}$ mice, we analyzed the hepatic copper metabolism gene expression patterns in 4- and 7-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. We observed that *Mt1* and *Cp* were up regulated and *Commd1* was down regulated in *Npc1*$^{-/-}$ mice. MT1 is a metal-induced protein that has the protective function of storing excess intracellular copper to detoxify cells[35,49]. Therefore, this result suggests that the mechanisms of protection against copper overload were activated early and maintained, at least until 7 weeks of age in *Npc1*$^{-/-}$ mice. Studies have shown that CP is a copper-dependent ferroxidase necessary for iron efflux of the liver that contributes to more than 70% of total plasma copper content[42,50,51]. Therefore, an alteration in hepatic copper metabolism may affect the synthesis of holo-CP and thus also adversely affect iron metabolism. *Cp* up-regulation in the

liver of *Npc1*$^{-/-}$ mice was correlated with an increase in plasma copper, which is associated with the increase of plasma CP in 7 and 8 weeks of age *Npc1*$^{-/-}$ mice[52]. However, it is not clear how CP activity could be increased if ATP7B function is impaired, as has been previously proposed in U18666A treated cells[26,30,53]. Our results indicated that in *Npc1*$^{-/-}$ mice the biosynthetic function of ATP7B is not altered, but rather that ATP7B-dependent copper transport to the bile is. The copper chaperone ATOX1 and COMMD1 participate with ATP7B in the biliary copper excretion process and consistently, a functional defect in these proteins leads to liver copper overload[25,54]. Although we did not observe transcriptional changes of *Atp7b* or *Atox1* in *Npc1*$^{-/-}$ mice, we found a significant decrease of *Commd1* expression in 4-and 7-week-old *Npc1*$^{-/-}$ animals. In agreement with this finding, reduced expression of *Commd1* was described at 7 and 8 weeks of age in *Npc1*$^{-/-}$ mice[27]. These results suggest that the early transcriptional alteration of *Commd1* may affect the vesicular traffic of ATP7B and copper into the bile duct. Thus, an adequate interplay among COMMD1, ATP7B and NPC1 may be necessary for an early and controlled copper efflux under physiological conditions. In fact, *in vitro* studies using U18666A treated hepatoma cell line suggest ATP7B function was impaired due to the disruption of the late endosome-to-TGN transport[26].

Interestingly, the higher copper content in liver and the lower copper in bile observed in *Npc1*$^{-/-}$ mice has been described in other genetics models like Wilson's disease mice (*Atp7b*$^{-/-}$)[55] and in the canine models of copper toxicosis (Bedlington terrier) caused by COMMD1 mutations[56,57]. These models have hepatic copper excess in common, which is caused by disorders in biliary excretion of the metal. However, while in *Npc1*$^{-/-}$ mice copper accumulation in the liver is twice that of control mice, in Wilson's disease models and in the canine models of copper toxicosis liver copper accumulation is 10 or 100 times higher[58]. The extent of copper accumulation in the liver of *Npc1*$^{-/-}$ mice is similar to that described by Muller et al. in 129/SvEv mice supplemented with 6 mM of copper in drinking water for one month[59]. We found a difference in the amount of copper accumulated in the livers of *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$, which suggests that biliary excretion requires the functional integrity of NPC1, ATP7B and COMMD1. Moreover, the higher copper content in the liver and plasma of *Npc1*$^{-/-}$ mice and the lower bile copper content observed in the *Npc1*$^{-/-}$ mice strongly suggests that the flux of copper from the liver into the plasma and into the bile are altered in *Npc1*$^{-/-}$ mice. The higher copper content in the plasma of *Npc1*$^{-/-}$ mice may be an example of an adaptive mechanism, which protects the liver against copper toxicity. Also, it is clear that copper transport mechanisms involved in the adaptation of increased hepatic copper storage require the activity of intracellular trafficking pathways which depend on the integrity of the NPC1 protein.

In order to understand the molecular mechanisms associated with alterations of hepatic iron metabolism in *Npc1*$^{-/-}$ mice, we analyzed the hepatic iron metabolism gene expression patterns in 4- and 7-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice. Iron metabolism at the cellular level is self-regulated through iron-dependent changes in the abundance of *Ft*, which sequesters excess iron and transferrin receptors controlling iron uptake[60,61]. When iron levels are low, *TfR* mRNA is stabilized, more receptor is synthetized, and iron uptake increases, while *Ft* mRNA is masked, FT synthesis and iron storage declines[60]. During the pre-weaning period, a significant reduction in iron liver and plasma content were detected in *Npc1*$^{-/-}$ mice compared to *Npc1*$^{+/+}$ mice, and these differences were maintained in 7-week-old animals. These results suggest that iron uptake and/or efflux processes are affected in *Npc1*$^{-/-}$ mice. The lower iron liver and plasma content in *Npc1*$^{-/-}$ mice suggests a diet deficiency. However, under our experimental conditions, all animals were administered the same diet. In particular, the diet given during the post-weaning period contained 45 mg Fe/kg, an amount clearly over the minimum commonly used to induce dietary iron deficiency (<8 mg Fe/kg)[62]. Thus, our results suggest that *Npc1* mutations cause iron deficiency, as has been showed in other mouse models of lysosomal disorders (murine glangliosidoses)[63], possibly affecting the uptake of the metal at the intestinal epithelium level. Iron intestinal epithelium levels should be analyzed to determine if the lower iron content in livers of *Npc1*$^{-/-}$ mice is due to lower intestinal absorption of this metal. It is known that iron liver uptake occurs principally through endocytosis mediated by TF/TFRC, these results suggest that the lower iron content in the livers of *Npc1*$^{-/-}$ mice may have been due, at least in part, to the abnormal recycling of TF/TFRC, a possibility previously proposed in CHO cell lines containing the *Npc1* mutation[15]. In addition, the amount of hepatic iron may be also affected by the expression of *Ft*. In fact, earlier studies using polyclonal and monoclonal antibodies showed low expression of H and L FT isoforms in various tissues from NPC patients[17, 64]. The authors suggested that iron is sequestered in lysosomes and cannot be recycled for FT synthesis in the *Npc1*$^{-/-}$ liver, nor can cholesterol and other lysosomal cargo[17].

Regarding the reduction of plasma iron in *Npc1*$^{-/-}$ mice , it is known that, ferroportin (Fpn) is a critical protein for the distribution of iron between tissues and its expression can be induced by iron and heme, and may be inhibited by hepcidin (Hepc) and inflammation[65,66,67]. Data from our study indicated an early and sustained reduction of *Fpn* in the liver of *Npc1*$^{-/-}$ mice, these results were correlated with lower iron content in plasma and increased expression of *Hepc* in the liver of *Npc1*$^{-/-}$ mice at 4 weeks of age. The decreased expression of *Fpn* in the liver of *Npc1*$^{-/-}$ mice, suggests a reduced iron efflux from the liver to the plasma on the *Npc1*$^{-/-}$ mice. Previous data shows that Hepc is the hormonal regulator of iron metabolism produced by the liver in mammals, which controls iron efflux to the plasma through the regulation of Fpn lysosomal degradation of the plasma membrane

of hepatocytes and enterocytes[68,69]. Taken together, liver and plasmatic iron content and iron genes expression results support the hypothesis that NPC1 is necessary for adequate and efficient management of cellular hepatic of this metal. However, further investigations are required to elucidate the mechanisms involved in the NPC1-dependent changes in gene transcription of homeostatic components of iron metabolism and their pathophysiological consequences.

Effects of copper intake restriction in the Niemann-Pick C murine model.

In order to reduce liver and plasma copper excess we provided 4-week-old *Npc1*$^{-/-}$ mice a copper-deficient diet for one or four weeks. The results indicate a fast (one week) hepatic and extra hepatic adaptive responsiveness to dietary copper deficiency in *Npc1*$^{-/-}$ mice, evidenced by a remarkable decrease of copper content in the liver, the same pattern was observed to a lower degree in bile and the heart. These changes were correlated with copper-related gene expression changes in the liver and cardiac phenotype alterations. The liver and heart copper content changes observed in *Npc1*$^{-/-}$ mice were similar to those described previously in wild type mice fed a copper-deficient diet in the third and fourth weeks of treatment[70]. Regarding the effect of a copper-deficient diet in the extra-hepatic organs, we observed a decrease in the left ventricular and interventricular wall thickness in *Npc1*$^{-/-}$ mice in the first week of the diet. Interestingly, FVB wild type mice fed with the same diet used in the present study, manifested an increase (cardiac hypertrophy) after four weeks of diet treatment[70]. These results suggest that *Npc1*$^{-/-}$ mice have a higher sensitivity to a copper-deficient diet compared to *Npc1*$^{+/+}$ mice and FVB wild type mice. Therefore, the opposite effect of copper decreasing on heart parameters found in *Npc1*$^{-/-}$ mice is probably due to genetic background. Also, previous data shows that genetic background has a strong influence on NPC disease expression from the Npc1-npc nih mutation[28]. However, further study is needed to explore why *Npc1*$^{-/-}$ mice may have manifested a different response. At 8-week-old we do not found significant differences in the copper content in the heart of the wild type mice fed a copper-deficient diet or a standard diet, and between *Npc1*$^{-/-}$ mice fed with the same diets. It is possible that the lack of change shown in our results may indicate an adaptive response in the wild type; however, we do not feel this is the case because, as shown in the literature[70], by 8 weeks the deteriorative effects of a copper-deficient diet on the heart are apparent. Furthermore, in looking at each case individually, we found that in the wild type group there were 3 outliers in the sample of 8 mice. If we remove these outliers from subsequent analysis, we find significant decreases in the copper content of the heart at 8 weeks, which is in concordance with the findings of other studies[70,71]. With respect to the *Npc1*$^{-/-}$ mice, again we feel that by 8 weeks the effects of the diet are such that we are unable to discuss an apparent adaptive response.

In order to understand the molecular mechanisms associated with alterations of hepatic copper metabolism in 4 weeks-old *Npc1*$^{-/-}$ mice, fed a copper-deficient diet; we analyzed the hepatic copper metabolism gene expression patterns in *Npc1*$^{-/-}$ mice fed a copper-deficient diet and *Npc1*$^{-/-}$ mice fed a control diet. We observed that *Mt1* and *Cp* were down regulated and these results were correlated with a reduction of liver copper content. Eight-week-old *Npc1*$^{-/-}$ mice presented high levels of copper in the liver and liver functional damage, as evidenced by the large increases in the levels of liver disease markers such as plasmatic alanine and aspartate aminotransferases, similar to those described previously in *Npc1*$^{-/-}$ mice[27,72,73]. Evidence supports the notion that copper excess favors oxidative stress and tissue damage[74,75]. In order to decrease hepatic copper content in *Npc1*$^{-/-}$ mice and to study the effect of a potential therapeutic intervention for NPC patients, we treated *Npc1*$^{-/-}$ mice with DPA, an effective and safe copper chelator that allows the removal of the excessive stored amounts and prevents further accumulation of copper. DPA is currently used for the treatment of Wilson disease[47,48,76]. Although *Npc1*$^{-/-}$ mice treated with DPA showed no total copper content differences in the liver our results indicated a hepatic adaptive response and that *Npc1*$^{-/-}$ mice presented a decrease in copper in bile and an improvement in their hepatic function according to the significant decrease of AST and ALT levels in plasma. However, further studies are required to understand the mechanism involved in DPA improvement of liver function in *Npc1*$^{-/-}$ mice. This result suggest that if the NPC patients present hepatic damage associated to hepatic copper dyshomeostasis, then oral DPA treatment may contribute to alleviate their liver damage.

In summary our results showed that *Npc1*$^{-/-}$ mice could mount an adaptive response to a copper-deficient diet allowing a partial recovery of hepatic, biliary and plasma copper levels. Furthermore, our findings indicated an improvement in hepatic function without effects on neurological function in *Npc1*$^{-/-}$ mice after treatment with DPA, however further studies are required to understand the mechanism involved in DPA treatment effects and to know if DPA can be as useful to NPC patients as it is for those with Wilson's disease.

Conflicts of interest

All the authors of this work declare that they have no conflict of interest.

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FIGURES LEGENDS

Figure 1. Altered copper and iron homeostasis in the *Npc1*$^{-/-}$ mouse model. Copper liver (A), iron liver (B), copper in plasma (C) levels; iron in plasma (D) levels; and copper in bile (E) levels, of four-, seven and eight-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice determined by AAS. Transcript levels of six copper metabolism genes (F) and six iron metabolism genes in liver (G), of four- and seven-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice were determined by qPCR. Each gene transcript level was normalized toward *Ppia* in the corresponding samples. The results show the mean of relative abundance of transcript value in relation to a maximum value (100%) of one gene. Data represent mean ± SEM from five biological replicates. Statistical analysis: Mann-Whitney U test U test, * p □ 0.05, n=5.

Figure 2. Effect of a copper-deficient diet in copper hepatic metabolism in the *Npc1*$^{-/-}$ mouse model. Copper liver (A), copper in plasma (B), and copper in bile (C) levels of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice at four, five and eight weeks old were determined by AAS. Transcript levels of six copper metabolism genes (D) in five-week-old *Npc1*$^{+/+}$and *Npc1*$^{-/-}$ mice were determined by qPCR. For each gene transcript level was normalized toward *Ppia* in the corresponding samples. The results show the mean of relative abundance of transcript value in relation to a maximum value (100%) of one gene. Data represent mean ± SEM from five biological replicates. Statistical analysis: Mann-Whitney U test U test, * p □ 0.05, n=5.

Figure 3. Effect of a copper-deficient diet on heart copper content and in the cardiac morphology in the *Npc1*$^{-/-}$ mouse model. Copper heart (A) level of five- and eight-week-old *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice fed with adequate (CuA) or deficient-copper diet (CuD) were determined by AAS. Quantitation of left ventricular wall thickness (B and E), left ventricular internal dimension (C and F) and interventricular septum diastolic dimension (D and G), of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice of seven- (upper panel) and five-week-old (bottom panel) were determined by echocardiogram M-mode. Data represent mean ± SEM. Statistical analysis: Mann-Whitney U test, * p □ 0.05, n=8.

Figure 4. Effect of D-penicillamine copper chelator on copper levels in the liver, bile and plasma of *Npc1*$^{-/-}$ mouse model. Copper liver (A), copper in bile (B) levels, and copper in plasma (C) levels of *Npc1*$^{+/+}$ and *Npc1*$^{-/-}$ mice, treated for four weeks with DPA, 100 mg/kg weight of mice/day dissolved in drinking water. Copper levels were determined by AAS. Data represent mean ± SEM. Statistical analysis: Mann-Whitney U test, * p □ 0.05, n=5.

Figure 5. Effect of D-penicillamine copper chelator in the transaminases levels in plasma of *Npc1*$^{-/-}$ mouse model. Plasma transaminase, ALT (A) and AST (B) activity; were determined by the standard photometric method, using Merck Microlab 100. Data represent mean ± SEM. Statistical analysis: Mann-Whitney U test, * p □ 0.05, n=5.

Figure 6. D-Penicillamine treatment revealed limited therapeutic effects on gait change in treated *Npc1*$^{-/-}$ mice. Intra–paw and inter-paw measurements, including (A) max contact area (cm^2) mean, (B) print length (cm) mean, (C) print area (cm^2) mean, (D) swing speed (cm/s) mean, (E) single stance (s) mean as well as (F) body speed (cm/s) mean were measured from control diet and D-Penicillamine treated 9-week-old *Npc1*$^{-/-}$ mice. RF: right front limb; RH: right hind limb; LF: left front limb; LH: left hind lime. The gait changes measured by the CatWalk^{TM} system as describe in "material and methods". Data represent mean ± SEM, n = 4.

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FIGURE 1

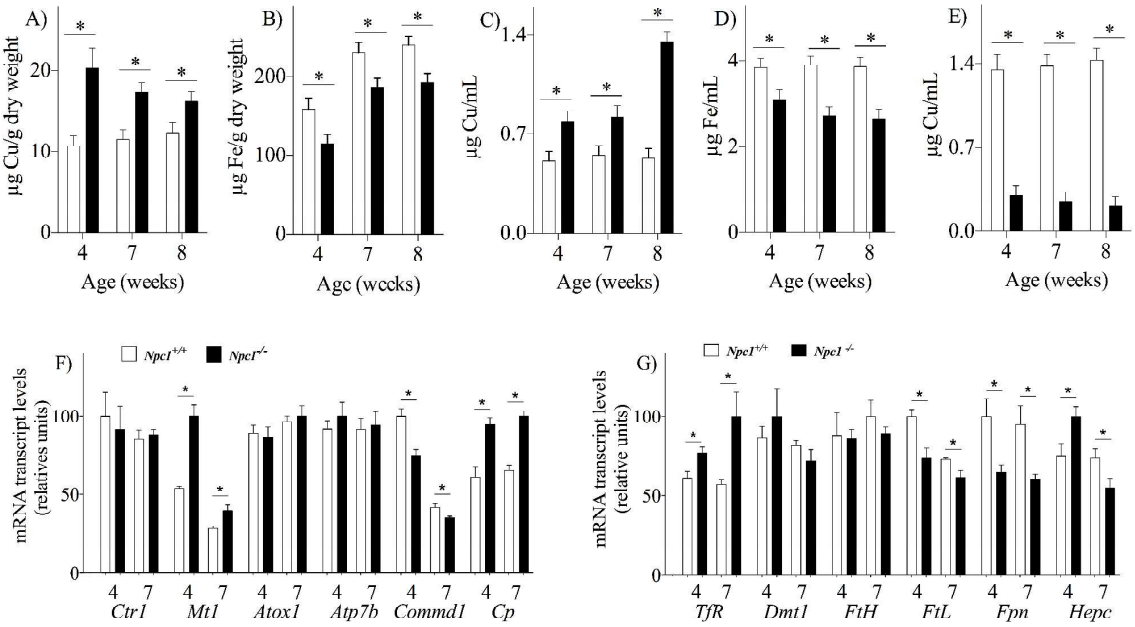

FIGURE 2

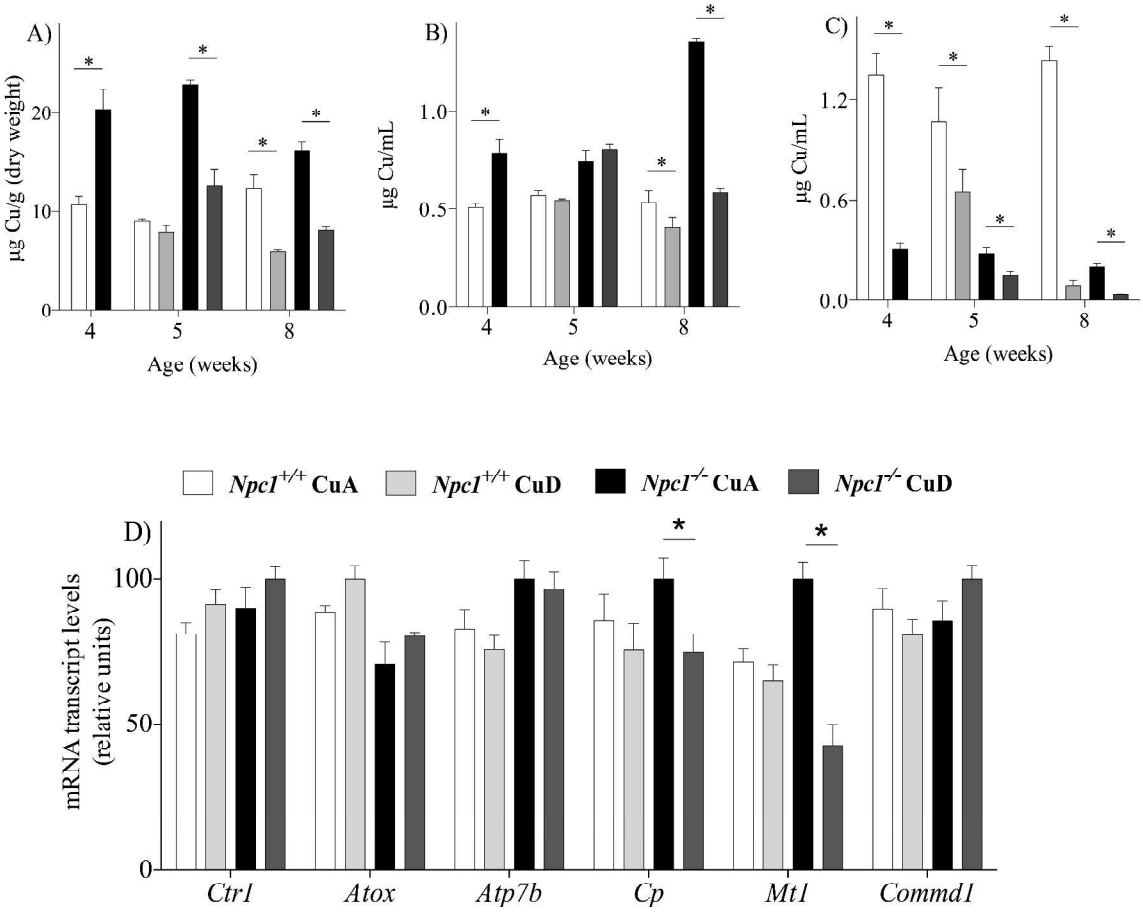

FIGURE 3

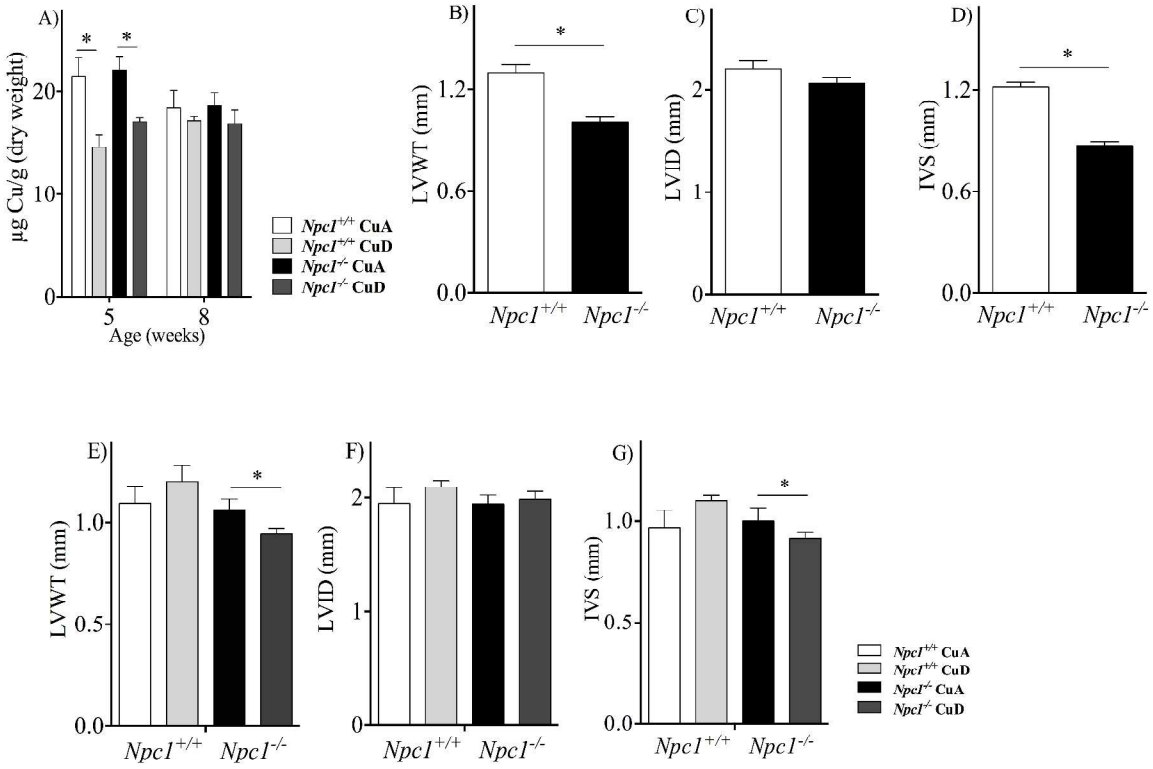

FIGURE 4

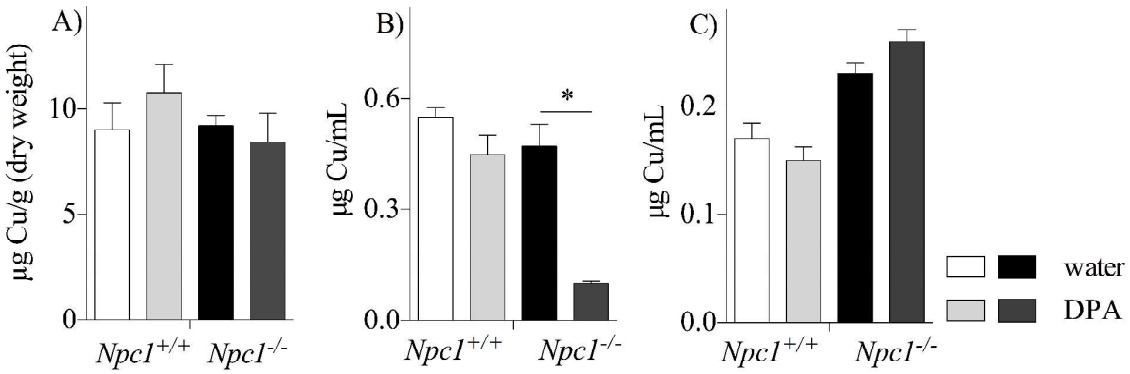

FIGURE 5

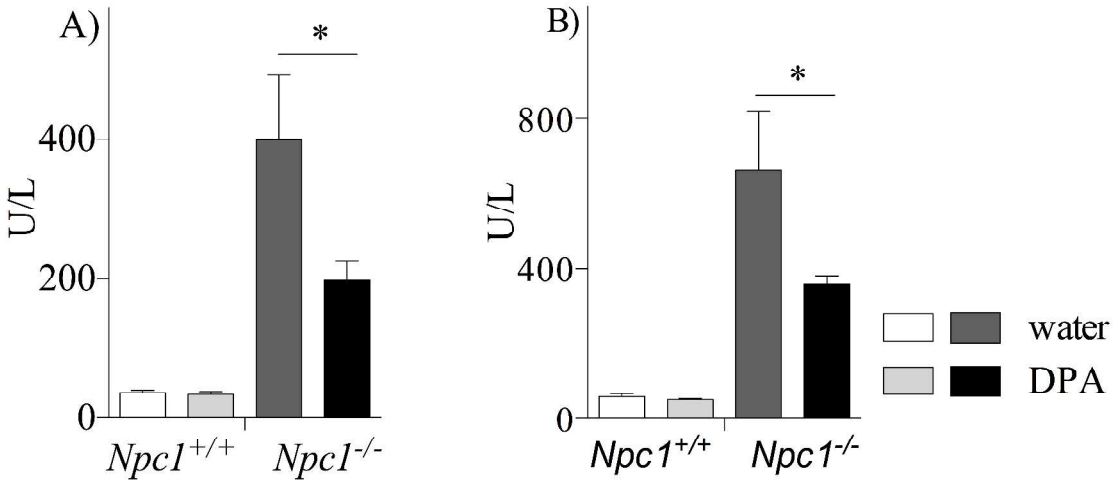

FIGURE 6