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The discrepancy between absence of copper deposition and presence of neuronal damage in brain of Atp7b$^{-/-}$ mice

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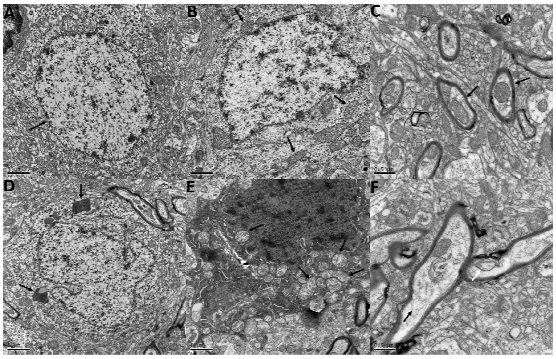

The neuronal damages were identified in the basal ganglia despite the absence of copper accumulation in the region.

Abstract

Wilson's disease (WD) is caused by mutations within the copper-transporting ATPase (ATP7B), characterized by copper deposition in various organs, principally the liver and brain. With the availability of Atp7b$^{-/-}$ mice, the valid animal model of WD, the mechanism underlying copper-induced hepatocyte necrosis has been well understood. Nonetheless, little is known about adverse impact of copper accumulation on the brain in WD. Therefore, the aim of this study was to identify copper disturbances according to various brain compartments and further dissect the causal relationship between copper storage and neuronal damage using Atp7b$^{-/-}$ mice. Copper levels in the liver, whole brain, brain compartments and basal ganglia mitochondria of Atp7b$^{-/-}$ mice and age-matched controls were measured by atomic absorption spectroscopy. Delicate electron microscopic studies on hepatocyte and neurons in the basal ganglia were performed. Here we further confirmed the remarkably elevated copper content and abnormal ultrastructure findings in livers of Atp7b$^{-/-}$ mice. Interestingly, we found the ultrastructure abnormalities on neurons of the basal ganglia of Atp7b$^{-/-}$ mice, whereas copper deposition was not detected in the whole brain, even within the basal ganglia and its mitochondria. The disparity provided the new understanding of neuronal dysfunction in WD, and strongly indicated that copper might not be the solely causative player and other unidentified pathogenic factors could enhance copper toxic effect on neurons in WD.

Keywords: Wilson's disease· Atp7b$^{-/-}$ mice· Copper homeostasis· Ultrastructure feature

Introduction

Wilson's disease (WD) is a severe copper metabolism disorder caused by mutations within *ATP7B*, which encodes the copper-transporting ATPase. The protein is mainly expressed in the liver, [1] where it performs two vital physiologic functions, including biliary copper excretion and synthesis of holoceruloplasmin. Accordingly, when ATP7B function is disrupted, copper gradually accumulates in the liver and other organs thereby causing complex symptoms. It principally encompasses hepatic, neurological and psychiatric disturbance or complicated combinations of these malfunctions.

Currently, several animal models for WD have been successfully established, including the Long-Evans Cinnamon (LEC) rats and Toxic milk mice (*tx* mice) have also been characterized in details. [2, 3] Although they share some similarities with WD patients, there are still some limitations residing in these models, such as the incomplete loss-of-function of Atp7b in *tx* mice [4] and various liver pathologies exist between LEC rats lineages due to involvement of other likely candidate genes. [5] Consequently, the genetically engineered $Atp7b^{-/-}$ mice eluding from the above shortcomings were generated and reckoned as an excellent rodent model for probing into the consequence of copper overload in the liver. [6, 7]

Apart from hepatic insufficiency, the nervous system is usually implicated in WD patients as well. Some neuroimaging findings show that the most common lesion in the brain is observed in the basal ganglia. [8-10] Thus, copper disturbance in the brain is postulated to be not ubiquitous over the course of WD. Therefore, it is necessary to re-analysis copper concentrations on the basis of various brain regions of $Atp7b^{-/-}$ mice.

Additionally, the accumulating evidences indicated that other trace elements,

other than copper, could overload in the brain and disrupt neuronal homeostasis in WD.[11, 12] Thus, the definite correlation between copper overload and brain lesions deserves further investigation. To better verify this perspective, we specially adopted the mutant mice, displaying no significant difference in the copper levels of the basal ganglia, to investigate whether their neuron homeostasis is disturbed. Altogether, the aim of this study was to evaluate whether there are significant differences in copper content of various brain regions between the two genotyped mice, and obtain some novel understandings of causative factors responsible for neuronal dysfunction in WD.

Experimental details

Animal husbandry and preparation of samples

The $Atp7b^{-/-}$ mice were kindly donated by Prof. Svetlana Lutsenko from Johns Hopkins University, and generation procedure has been explicitly depicted in the previous literature. [6] The mice were housed at Animal Care Facility of Shanghai Medical College and kept under standard conditions. $Atp7b^{+/+}$ mice, being a sibling line of $ATP7b^{-/-}$ mice, were used as normal controls. The liver and brain were immediately separated after being euthanized. The brain samples were divided into three discrete areas consisting of cerebral cortex (CX), basal ganglia (BG) and cerebellum (CB). CX encompasses frontal, temporal and parietal cortices; BG consists of caudate nucleus, putamen and globus pallidus. These studies were approved by the Institutional Animal Care and Use Committee of China and in accordance with the Guidelines on Animal Experiments of Shanghai Medical College.

Qualification of copper in the liver，whole brain and diverse brain compartments

The samples were dried in a vacuum oven at 56°C until water was thoroughly evaporated. After the acquisition of dried tissues weight, the samples were burned into ashes in the electric furnace of 500°C and then dissolved in concentrated HNO_3 in an induction cooker at 150°C until the solution became transparent. Copper content in the solution was determined by polarized atomic absorption spectrophotometry (AAS) using a Hitachi Z-5000 spectrophotometer. Finally, copper levels were presented as μg/g tissue (dry weight).

Copper content determination in mitochondrial isolation from the liver and brain homogenates

The Mitochondria Isolation Kit for Tissue (Pierce, America) was applied to isolate mitochondria from the liver tissues and basal ganglia. Copper levels in diverse mitochondrial preparations were analyzed via AAS after wet washing of mitochondria fraction with concentrated HNO_3.

Electron microscopy examinations on hepatocyte and neuron of basal ganglia

Electron microscopy was performed in 7 $Atp7b^{-/-}$ mice and 7 $Atp7b^{+/+}$ mice aged 10 months. The tissues were rinsed by the pre-cooling (4°C) normal saline and carved to the blocks (≤ 3mm³). The blocks were in turn immersed in 2.5% glutaraldehyde for fixing (≥ 2 hours), for rinsing 3 times, 1% OsO4 for post-fixing and 3 times again rinsing. After the completion of fixation, they were transferred to the ethanol and acetone for dehydrating and Epon for embedding. For thoroughly curing, the tissues were successively put in oven at 37°C for 24 hours, 45°C for 12 hours and 60°C for 48 hours. With the availability of Leica ultramicrotome, the ultrathin sections were double-obtained and then were stained with uranyl acetate and chromatic acid lead. Ultimately, the section were viewed and photographed by

the means of JOEL transmission electron microscope.

Statistical analyses

Copper levels were expressed as mean ± SD. Student's t-test was used to assess whether statistically significant variations of copper concentrations existed between the two genotyped mice in liver and three main brain compartments. Significant differences were considered when the *p* value < 0.05. In order to avert the measurement error, each sample was determined two times.

Results

The significantly increased copper levels in the liver is in agreement with the abnormal electron microscopic findings on the hepatocyte

In order to assure that our bred animal model were successful, we analyzed hepatic copper concentrations of $Atp7b^{-/-}$ and $Atp7b^{+/+}$ mice at aged 1, 3, and 10 months. The hepatic copper data demonstrated the statistically significant copper elevations in $Atp7b^{-/-}$ mice compared with age-matched controls (**Table 1**), being in accordance with the previous report.[7] Comparison of hepatic copper content of $Atp7b^{-/-}$ mice at different ages revealed the discontinuous trend in copper accumulation. The magnitude of copper deposition correlated with the copper-induced hepatic necrosis other than the length of exposure time. The highest copper level was detected at 3 months of age and the concentration values of $Atp7b^{-/-}$ mice were over 36 times higher than those of age-matched $Atp7b^{+/+}$ mice. Then the elevated trend of copper decreased to 34-fold at 10-month mice.

As shown in **Figure 1**, the typical hepatic ultrastructure alterations of WD patients were well-preserved in our mice model. Some cell compartments, including mitochondria and lysosome, were responsive to elevated copper. Specifically, noted mitochondrial alterations and high electron-dense granulation

accumulations in lysosomes were comprehensively observed in the livers of all the 7 Atp7b$^{-/-}$ mice (**Figure 1: C, D**). Meanwhile, the extensive ultrastructure alternations were not observed in the controls (**Figure 1: A, B**).

Ultrastructure abnormalities on neurons of ATP7b$^{-/-}$ mice exist prior to the abnormal copper metabolism

In order to specifically dissect whether copper homeostasis in the whole brain and several brain compartments of Atp7b$^{-/-}$ mice were affected by the abnormal ATP7B protein function, we determined copper levels between the two genotyped mice. At variance with copper disturbance in the liver, the obvious difference was not observed between Atp7b$^{-/-}$ and Atp7b$^{+/+}$ mice at 1 and 3 months of age. Taking the gradual deposition trend of copper into account, we further compared two groups of mice up to 10 months for the sake of eliminating the possibility of the inadequate observation time, and there were after all not statistically significant changes between the two genotyped mice (**Table 2**).

Given that the previous report showing basal ganglia was the most vulnerable brain region in WD,[13] the delicate electron microscopy studies were employed to explore whether changes in the neuronal ultrastructure resembled those of liver. Strikingly, the dramatic changes in neurons were observed (**Figure 2: D, E, F**) whereas the electron microscopy remained normal in 7 age-matched Atp7b$^{+/+}$ mice (**Figure 2: A, B, C**). Except for the clear similarities in some ways, microtubule and microfilament also exhibited the loose conformation.

The preliminary mitochondria abnormalities in the basal ganglia neuron are not correlated to copper deposition

Although copper concentrations were normal in the basal ganglia of Atp7b$^{-/-}$ mice, several cellular compartments in neuron, especially mitochondria, displayed

structural peculiarities. These gross mitochondrial impairments were similar to the findings in livers of WD patients and ATP7b$^{-/-}$ mice, which were thought to be specific and an early hallmark. Moreover, massive studies demonstrated that mitochondria could be the first responder under an imbalanced copper condition, and these specific structure changes are among the earliest detectable pathological features in WD. Thus, we further determined copper concentrations in mitochondria of livers and basal ganglia regions between two genotyped mice. Being paralleled to copper abnormalities in livers, the intra-mitochondrial copper levels were significantly high in Atp7b$^{-/-}$ mice aged 10 months (**Figure 3**). Unexpectedly, the mitochondrial copper concentrations in the basal ganglia still displayed no significant difference (**Figure 3**).

Discussion

The disrupted copper metabolism and resultant depositions in some organs are clinical hallmark in WD due to the inactivation of copper transporter ATP7B protein. Liver dominates the unique position in understanding the overview of WD since it is the earliest affected organ. Thus we determined the copper levels and ultrastructure features of liver in our bred mice so as to ensure the accuracy of subsequent research on brain sections. Definitely, our study is in agreement with the previous result. [7] Moreover, copper levels in the liver at three distinct stages further clued that it might be released from necrotic hepatocytes as disease progressed.

The clinical picture of WD is wide, and understanding of various disease presentations is important. In WD patients, the neurological manifestations generally appear later than the hepatic abnormalities[14] and are thought to be secondary to hepatic insufficiency. The neurological profile mainly encompasses

cerebral-related, cerebellar and pyramidal manifestations.[15] The impaired brain regions in WD are always closely associated with excess copper deposition.[16] Apart from the basal ganglia, known as the most susceptible region in WD patients, the cerebellum and cortical cortex are also implicated in the advanced course.[17] In view of the scarce brain samples of patients, Atp7b$^{-/-}$ mice became the essential tool for helping to unveil copper imbalance in the brain sections. In order to facilitate the development of animal models, we analyzed and compared the copper levels between Atp7b$^{-/-}$ and Atp7b$^{+/+}$ mice according to three distinct brain regions. In the present study, copper concentrations of various brain regions in Atp7b$^{-/-}$ mice were not significantly different from those in Atp7b$^{+/+}$ mice till 10 months aged. This result appeared to be at variance with findings in the livers.

To preclude whether there were the elementary interspecies differences in brain copper metabolism, it is necessary to compare Atp7b$^{-/-}$ mice with the two other models, LEC rats and *tx* mice. Although there are clear similarities in hepatic phenotype among them, brain abnormalities in Atp7b$^{-/-}$ mice are less prominent than the two latters. [2, 3] LEC rats not only show significant copper accumulation in the brain compared to LEA rats, but occasionally present with neurological symptoms at aged between 8 and 12 months.[2] Contrarily, there is the only report roughly suggesting that copper content in the whole brain of Atp7b$^{-/-}$ mice significantly increase so far. [6] The following reasons might explain the diversity.

Firstly, in rats, Borjigin and colleagues discovered a spice variant,[18]pineal night-specific ATPase (PINA), being selectively produced from the promoter downstream of exon 8. Therefore, this variant, if also existing in Atp7b$^{-/-}$ mice, might be insusceptible to abnormal spice within exon 2. Presumably, the presence of the similarly alternative ATP7B variant could partially function in exporting

copper. Secondly, more and more studies demonstrated that ATP7A protein, one homologous copper-transporting ATPase, could compensate for the complete loss of ATP7B function in Atp7b$^{-/-}$ mice. [19-21] Overall, the above modifiers might be, at least in part, involved in the regulatory process of copper metabolism in Atp7b$^{-/-}$ mice.

It is widely thought that excess copper storage play essential role in the pathogenesis of WD. Because of the exhausted capacity to sequestrate copper in the liver, massive copper are released from copper-loaded hepatocytes and overload in the brain and other organs in succession. Copper is the dominating source of free-radical production and causes cell necrosis in liver.[22] Thus the complicated neurological picture of WD is probably closely associated with copper accumulations in diverse brain regions. Nonetheless, some conflicting findings cast doubts on the theory that copper is the solely causative factor bringing about the pathologic lesions of brain. Through investigating the copper distribution in the various brain compartments of patient died for WD, Faa and colleagues have demonstrated that the abnormal copper distribution is inconsistent with the symptom-related brain lesions, and the basal ganglia seems relatively normal, which determines the common neurologic symptoms in WD.[12] This discrepancy strongly stressed the existence of other players aggravating the copper toxicity in the brain. The paucity of brain autopsy specimens of WD patients further hinders the dissection into definite relation between copper deposition and neuronal impairment. Thus, an available Atp7b$^{-/-}$ model helps to determine the complex mechanism responsible for neuronal dysfunction or death in WD. Despite some studies have yielded supportive evidences for copper accumulation in some brain regions, they could not yet exclude the hypothesis that other factors also affect the

neurological profile of WD. In order to explicitly elucidate the correlation, we especially selected the 10-month aged mice to observe neuronal ultrastructure changes. The interesting finding that the abnormal microscopy alternations existed in neuron with the absence of copper overload was identified. This result further supports the aforementioned hypothesis.

The healthy ATP7B protein has dual play, except for excreting the excessive copper into bile, biosynthesis of holoceruloplasmin is another importantly physiological process. Copper overload in WD merely refers to the disrupted copper excretion. However, whether the drastic reduction of holoceruloplasmin could be involved in the pathogenesis of WD remained unclear. Holoceruloplasmin is a multicopper oxidase being tightly associated with iron metabolism, and on the molecular level iron and copper metabolism are closely interacted. [23] Therefore, some reports on the disrupted iron metabolism in livers of WD patients have been released. [11, 24] Moreover, researchers have detected that some WD patients manifested as the eccentric hypointensity on T2-weighted images and reckoned that this rare appearance could be due to the existence of paremagnetic effects of some trace elements, for instance iron and manganese. [8] These findings further demonstrated that other causative factors might participate in the pathogenesis of WD except for copper. Besides, patients with WD at the later stage often presented with the severe portal-systemic encephalopathy, thus chronic liver disease could enhance copper toxic role as well. Certainly, we only discovered the disparity at present and intensive study need to be performed to identify other exact players being involved in WD pathogenesis.

Conclusions

This study firstly identified the causal relationship between copper and

neuronal lesions in the Atp7b$^{-/-}$ mice in detail. The discrepancy that abnormal neuronal alternations occurred in the basal ganglia with the absence of copper deposition was detected. This disparity strongly indicated that copper might not be the solely causative player and other unidentified morbific factors could enhance copper toxic effect on neurons in WD. In addition, compared with copper metabolism in brain of Atp7b$^{-/-}$ mice, the noted copper metabolism profile in the liver suggested that the rodent model is the valid and excellent model to uncover the liver pathogenesis of WD.

Disclosure

The authors report no conflicts of interest.

Acknowledgments

The authors sincerely appreciated Prof. Svetlana Lutsenko for providing the precious animal model of WD. This work was supported by grants from the National Natural Science Foundation of China (81125009 and 30971013) and State Key Basic Research Program of China (2012CB932403).

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Legends of Figures

Figure 1. Ultrastructure features of livers in 10-month Atp7b$^{+/+}$ and Atp7b$^{-/-}$ mice. A: normal morphology of mitochondria (solid arrows) and nucleus (hollow arrow) in Atp7b$^{+/+}$ hepatocyte; B: abnormal electron-dense granulation in lysosome being not observed in Atp7b$^{+/+}$ hepatocyte; C: vacuolization of massive mitochondria in Atp7b$^{-/-}$ mutant hepatocyte (solid arrows); D: extensive electron-dense granulation existing in abnormal lysosome of Atp7b$^{-/-}$ mutant hepatocyte (solid arrows). Original magnifications: 5000 (A, C); 20000 (B, D).

Figure 2.Ultrastructure features of neurons in the basal ganglia region of 10-month Atp7b$^{+/+}$ and Atp7b$^{-/-}$ mice. A: normal nucleus inside Atp7b$^{+/+}$ neuron (solid arrow); B: normal mitochondria inside Atp7b$^{+/+}$ neuron (solid arrows); C: normal ultrastructure of Atp7b$^{+/+}$ axons (solid arrows); D: lipofuscin accumulation in the perinuclear area of Atp7b$^{-/-}$ mutant neuron (solid arrows); E: vacuolization of massive mitochondria in Atp7b$^{-/-}$ mutant neuron (solid arrows); F: the occurrence of loose conformation of microtubule and microfilament (solid arrow), and swollen mitochondria (hollow arrow) in the malformed axons (much larger in diameter and irregular shape) of Atp7b$^{-/-}$ mutant neurons. Original magnifications: 10000 (A, B, D, E); 20000 (C, F).

Figure 3. Copper content in mitochondria of the liver and basal ganglia of 10-month Atp7b$^{+/+}$ and Atp7b$^{-/-}$ mice. There is significantly difference of copper content in mitochondria of the liver of Atp7b$^{-/-}$ mice in comparison to aged-matched controls. However, in mitochondria of the basal ganglia, the significant difference was not detected between two genotyped mice. Significant difference is indicated

by asterisk (***p< 0.001).

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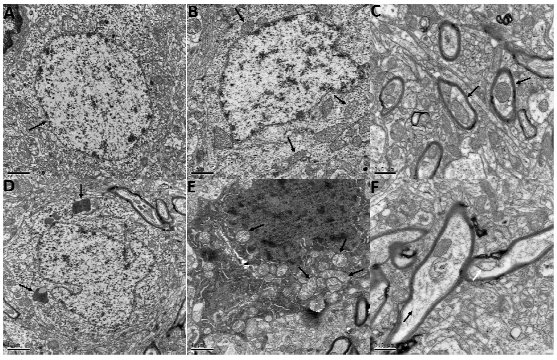
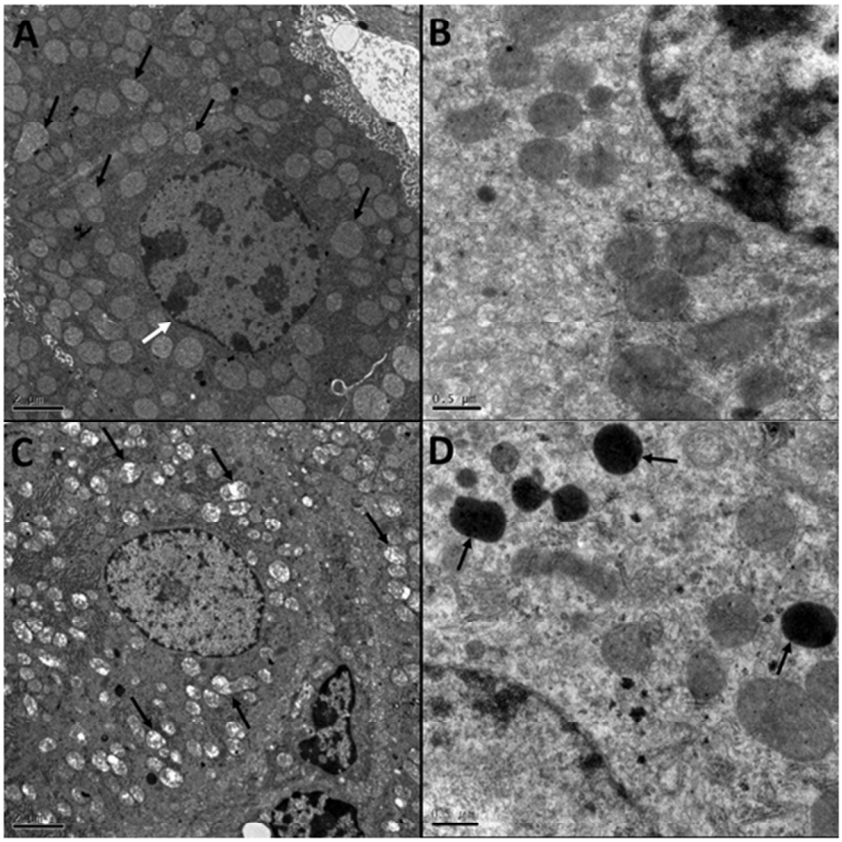


The neuronal damages were identified in the basal ganglia despite the absence of copper accumulation in the region.



Ultrastructure features of livers in 10-month Atp7b+/+ and Atp7b-/- mice. A: normal morphology of mitochondria (solid arrows) and nucleus (hollow arrow) in Atp7b+/+ hepatocyte; B: abnormal electron-dense granulation in lysosome being not observed in Atp7b+/+ hepatocyte; C: vacuolization of massive mitochondria in Atp7b-/- mutant hepatocyte (solid arrows); D: extensive electron-dense granulation existing in abnormal lysosome of Atp7b-/- mutant hepatocyte (solid arrows). Original magnifications: 5000 (A, C); 20000 (B, D).
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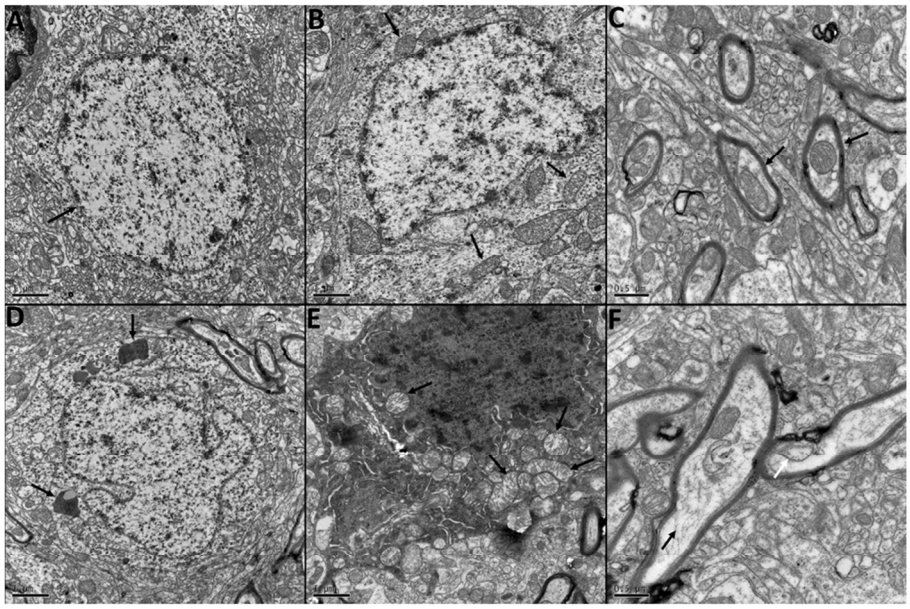

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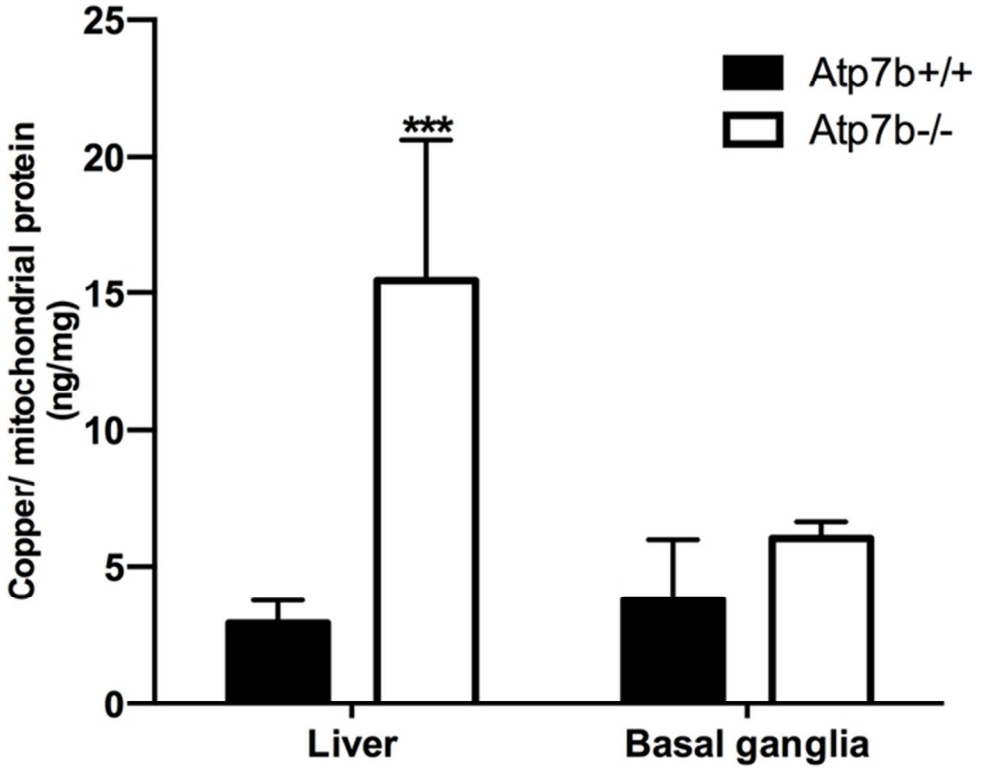

Copper content in mitochondria of the liver and basal ganglia of 10-month Atp7b+/+ and Atp7b-/- mice. There is significantly difference of copper content in mitochondria of the liver of Atp7b-/- mice in comparison to aged-matched controls. However, in mitochondria of the basal ganglia, the significant difference was not detected between two genotyped mice. Significant difference is indicated by asterisk (***p< 0.001).
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Table 1 Copper levels in livers of $Atp7b^{-/-}$ mice and controls

	One month	Three months	Ten months
$Atp7b^{+/+}$	21.2±2.0 (n=3)	24.1±3.7 (n=3)	18.4±1.4 (n=12)
$Atp7b^{-/-}$	575.5±27.9* (n=3)	874.5±9.4* (n=3)	627.0±48.4* (n=10)
Copper ratio ($Atp7b^{-/-}$/ $Atp7b^{+/+}$)	21	36	34

Copper levels are expressed as the mean concentration (μg/g dry weight) ± SD with number of animals in each group in parenthesis. The copper concentrations in $Atp7b^{-/-}$ mice were significantly different from controls during three distinct stages (* $p < 0.005$).

Table 2 Comparisons of copper levels in whole brain and distinct brain regions between the two-genotyped mice

Age(months)	Basal ganglia copper		Cerebellum copper		Cerebellum copper		Brain copper	
	$Atp7b^{-/-}$	$Atp7b^{+/+}$	$Atp7b^{-/-}$	$Atp7b^{+/+}$	$Atp7b^{-/-}$	$Atp7b^{+/+}$	$Atp7b^{-/-}$	$Atp7b^{+/+}$
1	35.1±5.7	30.4±7.4	20.8±7.2	20.1±2.0	45.3±14.3	40.0±12.0		
	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)		
3	35.5±9.5	25.2±2.3	17.1±1.7	17.4±1.8	26.8±0.7	41.4±7.8		
	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)	(n=3)		
10	30.7±2.7	39.8±4.1	27.5±2.8	22.5±3.0	49.9±7.0	33.7±4.8	32.9±3.0	29.9±2.6
	(n=10)	(n=11)	(n=10)	(n=11)	(n=10)	(n=11)	(n=6)	(n=4)

Copper levels are expressed as the mean concentration (μg/g dry weight) ± SD with number of animals in each group in parenthesis. The mean copper concentrations of three brain compartments and total brain were not significantly different between mutant mice and controls.