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Age-dependent Changes of Cerebral Copper Metabolism in *Atp7b*^{-/-} Knockout Mouse Model of Wilson's Disease by [⁶⁴Cu]CuCl₂-PET/CT

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Abstract

Copper is a nutritional metal required for brain development and function. Wilson's disease (WD), or hepatolenticular degeneration, is an inherited human copper metabolism disorder caused by mutation of *ATP7B* gene. Many WD patients present with variable neurological and psychiatric symptoms, which may be related to neurodegeneration secondary to copper metabolism imbalance. The objective of this study is to explore feasibility and use of copper-64 chloride ([⁶⁴Cu]CuCl₂) as a tracer for noninvasive assessment of age-dependence changes of cerebral copper metabolism in WD using an *Atp7b*^{-/-} knockout mouse model of WD and a positron emission tomography/computed tomography (PET/CT) scanner. Continuing from recent study of biodistribution and radiation dosimetry of [⁶⁴Cu]CuCl₂ in *Atp7b*^{-/-} knockout mice, PET quantitative analysis revealed low ⁶⁴Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice at 7th week of age, compared with the ⁶⁴Cu radioactivity in the brains of age and gender-matched wild type C57BL/6 mice, at 24 hour (h) post intravenous injection of [⁶⁴Cu]CuCl₂ as a tracer. Furthermore, age-dependent increase of ⁶⁴Cu radioactivity was detected in the brains of *Atp7b*^{-/-} knockout mice from 13th to 21th week of age, using the data derived from a longitudinal [⁶⁴Cu]CuCl₂-PET/CT study of *Atp7b*^{-/-} knockout mice with orally administered [⁶⁴Cu]CuCl₂ as a tracer. The findings of this study support the use of [⁶⁴Cu]CuCl₂-PET/CT as a tool for noninvasive assessment of age-dependent changes of cerebral copper metabolism in WD patients presenting with variable neurological and psychiatric symptoms.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of the paper

Keywords

copper metabolism; Wilson's disease; *Atp7b* gene; Neurodegeneration; Positron emission tomography; Copper-64 chloride

Introduction

Copper is an essential nutrient required for development and function of human brain [1, 2]. Wilson's disease (WD) is an inherited copper metabolism disorder caused by mutation of *ATP7B* gene encoding a copper-transporting P-type ATPase functioning as an efflux copper transporter [3–5]. The ATP7B efflux copper transporter is highly expressed in liver and plays an important role in delivery and biliary excretion of copper from liver. Malfunction of ATP7B efflux copper transporter causes accumulation of excess amount of copper ions in the liver, resulting hepatic metabolic changes and liver tissue damage. The ATP7B protein is also expressed in the brain and plays an important role in maintenance of cerebral copper homeostasis. Development of neurological and behavioral disorders in some of WD patients is likely related to disruption of copper homeostasis in the brain due to malfunction of ATP7B protein. The molecular mechanism of variable presentation of neuropsychiatric symptoms in WD patients remains unclear, largely due to lack of a tool for real-time assessment of changes of cerebral copper metabolism related to neurodegeneration within the brain of WD patients. Positron emission tomography (PET) is a useful technology for functional imaging of cerebral metabolic activity *in vivo* based on its high sensitivity and quantification capability. Recently, biodistribution and radiation dosimetry of [⁶⁴Cu]CuCl₂ in *Atp7b*^{-/-} knockout mice, a well-established mouse model of WD [6], was determined by PET/CT analysis [7–9]. Exploring feasibility and use of [⁶⁴Cu]CuCl₂ as a tracer for noninvasive assessment of changes of cerebral copper metabolism in WD, this study aimed to assess age-dependent changes of ⁶⁴Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice using PET/CT, following intravenous injection of [⁶⁴Cu]CuCl₂ as a tracer. Positron emitting 2-deoxy-2-[F-18]-fluoro-D-glucose ([¹⁸F]FDG) is a glucose analogue [10], which is widely used as a tracer for assessment of changes of cerebral glucose metabolism in neurological and psychiatric disorders with PET. In order to determine whether [⁶⁴Cu]CuCl₂ can be used as a tracer for assessment of cerebral copper metabolism, a sequential PET/CT imaging was conducted to compare biodistribution and cerebral [¹⁸F]FDG and ⁶⁴Cu radioactivity in mice intravenously injected with [¹⁸F]FDG and [⁶⁴Cu]CuCl₂ as a tracer, respectively. Continuing from recent studies of ⁶⁴Cu biodistribution and radiation dosimetry in the *Atp7b*^{-/-} knockout mice administered with [⁶⁴Cu]CuCl₂ intravenously [7] or orally [8, 9], further PET quantitative analysis was performed to measure age-dependent changes of ⁶⁴Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice. The data from this preclinical study support further development of [⁶⁴Cu]CuCl₂ as a new tracer for assessment of age-dependent changes of copper metabolism in WD patients presented with neuropsychiatric symptoms using a PET or PET/CT scanner.

Materials and Methods

Small animals and Radiopharmaceuticals

Balb/c mice were purchased from the animal resource center of the University of Texas Southwestern Medical Center (Dallas, TX). Free access to copper-adequate food and drinking water was provided routinely by animal housing facility, at UT Southwestern Medical Center at Dallas. The tracer [^{64}Cu]CuCl₂ was purchased from Washington University (St Louis, MO), which was produced via $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ on a biomedical cyclotron and supplied in a form of [^{64}Cu]CuCl₂ in 0.1M HCl solution. The specific activity of ^{64}Cu was 6.9 ± 2.5 Ci/ μmol . All small animal experiments were conducted according to the protocol approved by the Institutional Animal Care and Use Committee, UT Southwestern Medical Center, Dallas, TX.

[^{18}F]FDG PET/CT imaging

[^{18}F]FDG PET/CT imaging of Balb/c mice was performed using a method described previously [11, 12]. In short, Balb/c mice were anesthetized by inhalation of 3% isoflurane in 100% oxygen (3 L/min) at room temperature, using an isoflurane vaporizer (Summit Anesthesia Solutions, Salt Lake City, UT) and scanned using a Siemens Inveon PET/CT multimodality system (Siemens Medical Solutions). The mice were transferred to spread-supine position on the imaging bed and subjected to inhalation of 2% isoflurane in 100% oxygen (3 L/min) during the PET/CT procedure. After intravenous injection of [^{18}F]FDG (2 μCi (74 kBq)/g body weight) via tail vein, the mice were subjected to PET/CT imaging at 30 minutes and 2 h post injection (p.i.), respectively. Dynamic whole body data acquisition was started for 30 minutes with the head in the center of the field of view (FOV), followed by static imaging for 15 minutes at 2 h p.i. PET/CT images were reconstructed using the ordered subsets expectation maximization 3D algorithm (OSEM3D), and data were analyzed using the Inveon Research Workplace (IRW) software (Siemens) which allows fusion of CT and PET image volumes.

[^{64}Cu]CuCl₂-PET/CT imaging

[^{64}Cu]CuCl₂-PET/CT imaging of Balb/c mice was performed using a method described previously [7–9, 13]. Briefly, Balb/c mice were anesthetized by 3% isoflurane in 100% oxygen (3 L/min) at room temperature, using an isoflurane vaporizer (Summit Anesthesia Solutions, Salt Lake City, UT). The mice were positioned in a spread-supine position on the imaging bed and subjected to inhalation of 2% isoflurane in 100% oxygen (3 L/min) during the PET/CT procedure. PET/CT imaging was then initiated following intravenous injection of [^{64}Cu]CuCl₂ (2 μCi (74 kBq)/g body weight) diluted in a volume of 100 μL normal saline (0.9% sodium chloride) via tail vein. Dynamic whole body data acquisition was started for 30 minutes (5 minute frames) with the head in the center of the field of view (FOV), followed by static imaging for 15 minutes at 2 and 24 h p.i., respectively. PET/CT images were reconstructed using the ordered subsets expectation maximization 3D algorithm (OSEM3D), and data were analyzed using the Inveon Research Workplace (IRW) software (Siemens) which allows fusion of CT and PET image volumes.

Quantification of copper ions in mouse tissues by ICP-MS

To investigate correlation of ^{64}Cu radioactivity measured by [^{64}Cu]CuCl₂-PET/CT with copper ions present in mouse organs or tissues, mouse tissue copper ions in another group of Blab/c mice (n=5, female, 8 weeks of age) were quantified by Inductively coupled plasma mass spectrometry (ICP-MS) in a method similar to those previously described [9, 14, 15]. For sample preparation, mouse tissues were harvested after the Balb/c mice were euthanized under anesthesia. Tissues and organs were frozen for further study. Before copper concentration determination, tissues were collected and weighted. 200 μL of Fresh aqua regia (a mixture of nitric acid and hydrochloric acid nitric in a ratio of 1:3, tracer metal grade) purchased from Fisher scientific (Pittsburgh, PA) were added to these tissues, followed by heating to 80 $^{\circ}\text{C}$ for 20 min. Ultrasound was assisted to digested these tissues. After overnight digestion, all of the sample solutions were clear, without visible residues. The solution was diluted with Milli-Q water (Millipore gradient, USA) for ICP-MS determination. Copper analysis was performed with 7700 series ICP-MS instrument (Agilent technologies, CA). Calibration was performed using 6 to 8 standards and stable copper isotopes were measured with instrumental parameters. Copper concentration was recorded as ng/mg wet weight and values presented as means $\pm$ standard deviation (SD).

Quantification of ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice

Using the data acquired from our recent studies of biodistribution and radiation dosimetry of [^{64}Cu]CuCl₂ in *Atp7b*^{-/-} knockout mice [7, 8], ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice was analyzed using the Inveon Research Workplace (IRW) software (Siemens). In order to measure ^{64}Cu radioactivity in the different regions of a mouse brain, 9 regions of interest (ROIs) were defined manually on the PET/CT images with reference to an MR imaging-based atlas of mouse brain anatomy [16] as shown on Fig 2. Regions included were: ROI 1 for olfactory bulb (OB), ROI 2 for frontal cortex (FC), ROI 3 for posterior cortex (PC), ROI 4 for hippocampus (HC), ROI 5 for basal ganglia (BG), ROI 6 for thalamus (T), ROI 7 for middle brain (MB), ROI 8 for brain stem (BS), ROI 9 for cerebellum (CB). The quantity of ^{64}Cu radiotracer activity was obtained and recorded as a percentage of injected dose per gram tissue (%ID/g).

Statistical analysis

Friedman rank based ANOVA was used to test the difference in median cerebral ^{64}Cu radioactivity between *Atp7b*^{-/-} knockout mice and control C57BL/6 mice for each organ and for each time point (2 h and 24 h p.i., respectively). P value of less than 0.05 was considered as statistically significant.

RESULTS

Biodistribution of [^{18}F]FDG and ^{64}Cu radioactivity in mice

Sequential PET/CT imaging was performed to compare the biodistribution of [^{18}F]FDG and ^{64}Cu radioactivity in mice (Blab/c, n = 5, female, 8 weeks of age) after intravenous injection of [^{18}F]FDG and [^{64}Cu]CuCl₂, respectively. There was different biodistribution of ^{18}F FDG and ^{64}Cu radioactivity in Blab/c mice (Fig 1, Table 1). In contrast to abundant

[^{18}F]FDG in the brain of Balb/c mice injected with [^{18}F]FDG, there was relatively low ^{64}Cu radioactivity in the brain of Balb/c mice injected with [^{64}C]CuCl₂ intravenously. Moreover, regional analysis of the brain showed decreased cortical ^{64}Cu radioactivity at 24 h p.i. as compared to the ^{64}Cu radioactivity at 2 h p.i. (Fig 2, Table 2). With respect to all other brain regions, ^{64}Cu radioactivity at 24 h p.i. was higher than the ^{64}Cu radioactivity at 2 h p.i., except for unchanged ^{64}Cu radioactivity in the cerebellum (Fig 2, Table 2).

Copper concentration of mouse tissues determined by ICP-MS

Copper concentration in tissues of Balb/c mice (n = 5, female, 8 weeks of age) was quantified by ICP-MS. High concentration of copper ions was detected in the heart (7.49 ± 1.04 ng/mg), liver (6.51 ± 1.22 ng/mg), brain (5.66 ± 0.97), stomach (5.46 ± 1.57 ng/mg), and kidney (4.27 ± 0.93 ng/mg), compared with relatively lower concentration of copper ions in lungs (3.23 ± 0.88 ng/mg), muscle (2.31 ± 0.79 ng/mg), intestine (2.39 ± 0.49 ng/mg), and spleen (1.68 ± 0.68 ng/mg). High copper concentration of liver tissue was correlated with abundant hepatic ^{64}Cu radioactivity measured by [^{64}Cu]CuCl₂-PET/CT imaging (Table 1, Fig 3). Despite the high concentration of endogenous copper ions in brain tissue determined by ICP-MS, there was low ^{64}Cu radioactivity in the brain of Balb/c mice measured by [^{64}Cu]CuCl₂-PET/CT imaging (Fig 1 and Fig 3). These findings suggest that [^{64}Cu]CuCl₂-PET/CT is a useful tool for tracking copper fluxes *in vivo*, but is limited or not useful for indirect estimation of the concentration of endogenous copper ions in mouse tissues.

Decreased ^{64}Cu radioactivity in the brain of 7-week-old *Atp7b*^{-/-} knockout mice after intravenous injection of [^{64}C]CuCl₂

Quantitative analysis was performed to study ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice injected with $^{64}\text{CuCl}_2$ intravenously, using the data from recent study of ^{64}Cu biodistribution and radiation dosimetry in *Atp7b*^{-/-} knockout mice [7]. At 7th week of age, ^{64}Cu radioactivity at 24h p.i. in the brain of *Atp7b*^{-/-} knockout mice (0.34 ± 0.08 %ID/g) was significantly lower than the ^{64}Cu radioactivity in the brain of age-matched C57BL/6 mice (0.61 ± 0.09 %ID/g, $p < 0.03$) (Fig 4, Table 3). Moreover, whereas the brain ^{64}Cu radioactivity of control C57BL/6 mice increased between 2 and 24 h p.i., it was decreased between 2 and 24 h p.i. in the brain of *Atp7b*^{-/-} knockout mice. These results demonstrate the potential of $^{64}\text{CuCl}_2$ PET/CT imaging for tracking copper fluxes in the mouse brain.

Age-dependent changes of ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice determined by longitudinal PET/CT imaging after oral administration of [^{64}Cu]CuCl₂

In order to determine whether there are age-dependent changes of cerebral copper metabolism in *Atp7b*^{-/-} knockout mice, a quantitative analysis of ^{64}Cu accumulation in the brain of *Atp7b*^{-/-} knockout mice was performed using the data from recent longitudinal PET/CT imaging of *Atp7b*^{-/-} knockout mice after oral administration of [^{64}Cu]CuCl₂ [8, 9]. Age-dependent changes of ^{64}Cu radioactivity were identified in the brain of *Atp7b*^{-/-} knockout mice (n = 4, female) between 13 and 21 weeks of age (Table 4, Fig 5). At 13th week of age, ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice (0.05 ± 0.04 %ID/g) was lower than the ^{64}Cu radioactivity in the brains of control C57BL/6 mice (0.11

± 0.10 %ID/g, $p = 0.048$). Subsequently, there was an age-dependent increase of ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice. By 21th week of age, ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice (0.17 ± 0.09 %ID/g) was significantly higher than the ^{64}Cu uptake in the brain of age-matched control C57BL/6 mice (0.09 ± 0.05 %ID/g, $p = 0.042$). Moreover, quantitative analysis revealed significant increase of ^{64}Cu radioactivity in the region of hippocampus ($p = 0.042$), thalamus ($p = 0.002$), brain stem ($p = 0.001$), and cerebellum ($p = 0.014$) of the brains of *Atp7b*^{-/-} knockout mice, compared with those in the corresponding brain regions of control C57BL/6 mice (Table 4). No significant increase of ^{64}Cu radioactivity was identified in the regions of olfactory bulb ($p = 0.474$), frontal cortex ($p = 0.61$), posterior cortex ($p = 0.072$), and basal ganglia ($p = 0.256$) of the brains of *Atp7b*^{-/-} knockout mice, compared with ^{64}Cu radioactivity in the corresponding brain regions of control C57BL/6 mice (Table 4).

Discussion

Copper is required for development and function of brain, and copper metabolism imbalance secondary to malfunction of ATP7B efflux copper transporter causes neurodegeneration in WD [17, 18]. Some of WD patients present with symptoms of neurological and/or psychiatric disorders, while many of WD patients present with mainly symptoms of hepatic disease without significant symptoms of neuropsychiatric disorders. The cause of this phenotypic diversity in WD is not clear, that may be related to effect of *ATP7B* gene mutation on cellular localization and function of ATP7B efflux copper transporter and cellular copper tolerance [19] and effect of other modification factors [20]. Animals with genetic defects in *Atp7b* gene have been a remarkably useful tool in study of molecular mechanisms of WD pathophysiology. The *Atp7b*^{-/-} knockout mouse is a well-established mouse model of WD, developing hepatic pathology caused by accumulation of excess amount of copper ions in the liver [6, 21]. In contrast to neuroinflammatory and behavioral changes observed in toxic milk mouse model of WD [22, 23], no significant neuropsychiatric symptoms were observed in adult *Atp7b*^{-/-} knockout mice although neurological symptoms (tremor, ataxia, abnormal locomotor behavior) were observed in *Atp7b*^{-/-} knockout pups younger than 2 weeks of age and nursed by homozygous *Atp7b*^{-/-} mothers [21]. The cause of absence of neuropsychiatric symptoms in adult *Atp7b*^{-/-} knockout mice is not clear, that may be related to age-dependent changes of cerebral copper metabolism. Cerebral copper deficiency present at infant age is no longer present in the adult *Atp7b*^{-/-} knockout mice when there is improvement of availability and delivery of copper to the brain tissues. It remains to be determined whether old *Atp7b*^{-/-} knockout mice will develop neuropsychiatric symptoms when there is neuron loss caused by neurotoxicity of excess amount of copper ions accumulated in the brain tissues.

The data from sequential [^{18}F]FDG and [^{64}Cu]CuCl₂-PET/CT imaging of Balb/c mice (Table 1, 2 and Fig 1, 2) demonstrated the capability of ^{64}Cu to diffuse across the blood brain barrier, as previously described [24], supporting use of [^{64}Cu]CuCl₂ as a tracer for noninvasive assessment of cerebral copper metabolism *in vivo* by quantification of ^{64}Cu radioactivity using PET or PET/CT imaging. Our findings are in line with previous studies that applied [^{64}Cu]CuCl₂ as a tracer for investigation of cerebral copper metabolism in aging mice with autoradiography [25], study of changes of cerebral copper metabolism in a mouse

model of Menkes disease [26], as well as diagnostic imaging of traumatic brain injury [13]. To determine whether ^{64}Cu radioactivity measured by PET/CT is correlated with the concentration of endogenous copper ions in mouse tissues, copper concentration of mouse tissues was determined by ICP-MS and correlated with ^{64}Cu biodistribution in Balb/c mice on ^{64}Cu CuCl₂-PET/CT imaging (Fig 3). Intense ^{64}Cu radioactivity in the liver is correlated with a large quantity of hepatic endogenous copper ions measured by ICP-MS, but there is no direct correlation between relatively low cerebral ^{64}Cu radioactivity determined by PET/CT and high concentration of copper ions in brain tissues determined by ICP-MS (Fig 3). This suggests that ^{64}Cu CuCl₂-PET/CT measures dynamic fluxes and accumulation of ^{64}Cu in the mouse brain tissues, while ICP-MS measures total amount of endogenous copper ions in the mouse brain tissues, regulated by a network of copper transporters and chaperons [27–29]. It is important to study both of dynamic copper fluxes and quantity of endogenous copper ions in brain tissues in dissecting the role of disruption of copper homeostasis, either copper deficiency or accumulation of excess amount of copper ions, in pathophysiology of WD [14, 15, 30] and other neurological disease associated with copper metabolism disorders [28].

Continuing from recent biodistribution and radiation dosimetry study in *Atp7b*^{-/-} knockout mice injected with ^{64}Cu CuCl₂ intravenously [7], further PET quantitative analysis of current study demonstrated that ^{64}Cu radioactivity in the brains of 7-week-old *Atp7b*^{-/-} knockout mice was significantly lower than the ^{64}Cu radioactivity in the brains of age-matched control C57BL/6 mice (Table 3, and Fig 4). Low ^{64}Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice was likely related to sequestration of ^{64}Cu in the liver due to malfunction of Atp7b efflux copper transporter. On the other hand, low ^{64}Cu radioactivity in the *Atp7b*^{-/-} knockout mice at 7th week of age could also be related to reduced copper influx mediated by human copper transporter 1 or changes of copper transport mediated by other copper transporters or chaperons [27–29]. Compared with physiological ^{64}Cu radioactivity in the brains of C57BL/6 mice, low ^{64}Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice at 7th week of age may represent copper deficiency in the mouse brain tissues or decreased availability of copper from blood pool. It remains to be determined whether low ^{64}Cu radioactivity detected in the brain of *Atp7b*^{-/-} knockout mice at 7th week of age is associated with development of neurological and/or psychiatric disorders. Based on further PET quantitative analysis of the data acquired from recent longitudinal PET/CT imaging of *Atp7b*^{-/-} knockout mice after oral administration of ^{64}Cu CuCl₂ [8, 9], age-dependent changes of ^{64}Cu radioactivity was detected in the brain of *Atp7b*^{-/-} knockout mice [Table 4 and Fig 5]. There was gradual increase of ^{64}Cu radioactivity in the brain of *Atp7b*^{-/-} knockout mice from 13th to 21th week of age, likely due to increased availability of ^{64}Cu from blood pool when liver sequestration of ^{64}Cu is reduced or increased retention of ^{64}Cu in the brain tissues caused by malfunction of Atp7b copper efflux transporter. Increased ^{64}Cu radioactivity was detected in all of brain regions in *Atp7b*^{-/-} knockout mice by 21th week of age, including regions of cortex, basal ganglia, thalamus, midbrain and brain stem and cerebellum, with highest ^{64}Cu radioactivity detected in cerebellum [Fig 5]. It will be useful to correlate ^{64}Cu radioactivity detected by ^{64}Cu CuCl₂ PET/CT with abnormalities detected by other imaging modalities, such as abnormal fiber projection

between subcortical nuclei [31], for delineation of molecular mechanism of brain tissue damage in WD patients with neuropsychiatric disorders.

Addressing limitations of this study, the data of sequential [^{18}F]FDG and [^{64}Cu]CuCl₂ PET/CT imaging (Fig 1, 2, 3A) and mouse tissue copper concentration determined by ICP-MS (Fig 3B) were obtained in Balb/c mice, not in C57BL/6 mice used as a control group for assessment of age-dependent changes of cerebral ^{64}Cu radioactivity in *Atp7b*^{-/-} knockout mice. We used Balb/c mice for sequential [^{18}F]FDG and [^{64}Cu]CuCl₂ PET/CT imaging in this study because it was difficult to conduct tail vein injection in C57BL/6 mice and frequently there was leakage of radiotracer at tail vein injection site after multiple attempts of repeated tail vein injection. It was technically less challenging to conduct repeated tail vein injection in Balb/c mice than in C57BL/6 mice, therefore, we used Balb/c mice for sequential [^{18}F]FDG and [^{64}Cu]CuCl₂ PET/CT imaging in this study. There might be a small difference of [^{18}F]FDG and ^{64}Cu accumulation in the brain of Balb/c and C57BL/6 mice after intravenous injection of [^{18}F]FDG and [^{64}Cu]CuCl₂ respectively. This should not limit use of the data obtained from sequential [^{18}F]FDG and [^{64}Cu]CuCl₂ PET/CT imaging of Balb/c mice to determine feasibility and use of ^{64}Cu Cl₂ as a radiotracer for assessment of age-dependent changes of cerebral ^{64}Cu radioactivity in *Atp7b*^{-/-} knockout mice with PET/CT. The data of ^{64}Cu radioactivity measured from manually drawn mouse brain ROIs was subjected to spatial resolution error in view of small size of mouse brain and limited spatial resolution of a PET scanner. Additionally, biodistribution of ^{64}Cu Cl₂ in rodents might be different from that in humans considering possible difference of copper metabolism between rodents and human. Nevertheless, the findings of age-dependent changes of ^{64}Cu accumulation in *Atp7b*^{-/-} knockout mice support further study of age-dependent changes of cerebral copper fluxes in WD patients using [^{64}Cu]CuCl₂-PET/CT imaging. Radiation dosimetry of [^{64}Cu]CuCl₂ estimated from preclinical biodistribution and radiation dosimetry studies in rodents [7, 8, 32] and clinical use of [^{64}Cu]CuCl₂ as a radiopharmaceutical for diagnostic imaging of prostate cancer in human [33] support safe use of [^{64}Cu]CuCl₂ as a tracer for evaluation of cerebral copper metabolism imbalance in WD patients. In view of a tiny amount of copper ions (< 0.04 ng/ μCi of ^{64}Cu Cl₂) in a tracer dose of [^{64}Cu]CuCl₂, there is little concern of copper toxicity on neurons when a tracer dose of [^{64}Cu]CuCl₂ is administered for PET/CT imaging in human.

The data from this study demonstrated age-dependent changes of ^{64}Cu accumulation in the brains of *Atp7b*^{-/-} knockout mice, which are encouraging and support further investigation of age-dependent changes of copper metabolism in WD patients who suffer from psychiatric and neurological symptoms, using [^{64}Cu]CuCl₂-PET/CT imaging in conjunction with [^{18}F]FDG PET [34, 35] and MRI [35, 36]. Delineation of age-dependent changes of copper metabolism in WD patients is expected to contribute to a better understanding of molecular mechanism of neurodegeneration in WD. In summary, ^{64}Cu Cl₂-PET/CT is a promising new tool for assessment of the changes of cerebral copper metabolism in WD patients who present with psychiatric and neurological symptoms and may set the stage for individualized treatment of WD [17, 18, 37].

Conclusion

Age-dependent changes of cerebral ^{64}Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice were detected by PET/CT imaging after intravenous injection of [^{64}Cu]CuCl₂ as a tracer, supporting further development of [^{64}Cu]CuCl₂ PET/CT as a tool for noninvasive assessment of age-dependent changes of cerebral copper metabolism in WD patients.

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Fig 1. Biodistribution of ^{64}Cu and ^{18}F FDG in Balb/c mice by PET/CT imaging
A. Low ^{64}Cu radioactivity in the brain and intense ^{64}Cu radioactivity in the liver were visualized on the PET/CT image of Balb/c mice at 30 minutes p.i. of ^{64}Cu . In contrast, abundant physiological ^{18}F FDG radioactivity was visualized in the brain of Balb/c mice at 30 minutes p.i. of ^{18}F FDG. An open thick arrow points to the liver, whereas a thin arrow points to the brain. **B.** Biodistribution of ^{64}Cu and ^{18}F FDG in Balb/c mice by PET quantitative analysis, showing high ^{64}Cu radioactivity in the liver of Balb/c mice at 2 h p.i. of ^{64}Cu and abundant physiological ^{18}F FDG radioactivity in the brains of Balb/c mice at 2 h p.i. of ^{18}F FDG. %ID/g, percentage of injected dose per gram; p.i., post intravenous injection.

Fig 2. PET quantification of ^{64}Cu radioactivity in the brain of Balb/c mice after intravenous injection of $[^{64}\text{Cu}]\text{CuCl}_2$ as a tracer

A. Schematic presentation of 9 ROIs manually drawn within the region of brain on PET/CT imaging of Balb/c mice. **B.** ^{64}Cu radioactivity in the different regions of mouse brain by PET quantitative analysis. ^{64}Cu radioactivity in the cortex at 24 h p.i. was lower than the cortical ^{64}Cu radioactivity at 2 h p.i. In contrast, ^{64}Cu radioactivity in the other regions of brain at 24 h p.i. was higher than ^{64}Cu radioactivity in the brain at 2 h p.i.

ROI, region of interest; OB, olfactory bulb; FC, frontal cortex; PC, posterior cortex; HC, hippocampus; BG, basal ganglia; T, thalamus; MB, middle brain; BS, brain stem; CB, cerebellum. %ID/g, percentage of injected dose per gram; h, hour; p.i., post intravenous injection.

Fig. 3. Biodistribution of ^{64}Cu radioactivity determined by $[^{64}\text{Cu}]\text{CuCl}_2$ PET/CT imaging and concentration of copper ions in Balb/c mice measured by ICP-MS

Abundant ^{64}Cu radioactivity in liver and kidneys (A) corresponded with high concentration of endogenous copper ions determined by ICP-MS (B). In contrast, low ^{64}Cu radioactivity in the brain and heart tissue corresponded high concentration of copper ions in these tissues determined by ICP-MS. %ID/g, percentage of injected dose per gram; h, hour; p.i., post intravenous injection.

Fig. 4. Decreased ^{64}Cu radioactivity in the brains of *Atp7b*^{-/-} mice at 7th week of age compared with physiologic ^{64}Cu radioactivity in the brains of age-matched C57BL/6 mice

(A) Increased accumulation of ^{64}Cu was visualized in the liver on PET/CT images of *Atp7b*^{-/-} knockout mice after intravenous injection of [^{64}Cu]CuCl₂, compared with relatively low ^{64}Cu radioactivity in the liver of age-matched wild type C57BL/6 mice. (B) PET quantitative analysis demonstrated that ^{64}Cu radioactivity in the brains of *Atp7b*^{-/-} knockout mice was significantly lower than the ^{64}Cu radioactivity in the brains of control C57BL/6 mice at 24 h p.i of [^{64}Cu]CuCl₂.

ROI, region of interest; OB, olfactory bulb; FC, frontal cortex; PC, posterior cortex; HC, hippocampus; BG, basal ganglia; T, thalamus; MB, middle brain; BS, brain stem; CB, cerebellum. %ID/g, percentage of injected dose per gram; h, hour; p.i., post intravenous injection.

Fig. 5. Age-dependent changes of ^{64}Cu radioactivity in brains of $Atp7b^{-/-}$ knockout mice and control C57BL/6 mice by longitudinal PET/CT imaging after oral administration of $[^{64}\text{Cu}]\text{CuCl}_2$ as a tracer

A Age-dependent increases of ^{64}Cu radioactivity were detected in all brain regions in $Atp7b^{-/-}$ knockout mice at 24 h p.o. of $[^{64}\text{Cu}]\text{CuCl}_2$ as a tracer. Longitudinal PET/CT imaging was performed at 13th, 18th, and 21th week of age. **(B)** In contrast, age-dependent decrease of ^{64}Cu radioactivity were detected in all brain regions in C57BL/6 mice at 24 h p.o. of $^{64}\text{CuCl}_2$ as a tracer. Longitudinal PET/CT imaging was performed at 13th, 18th, and 21th week of age.

ROI, region of interest; OB, olfactory bulb; FC, frontal cortex; PC, posterior cortex; HC, hippocampus; BG, basal ganglia; T, thalamus; MB, middle brain; BS, brain stem; CB, cerebellum. %ID/g, percentage of injected dose per gram; h, hour; p.o., post oral administration.

Biodistribution of ^{64}Cu and [^{18}F]FDG in Balb/c mice by PET/CT after intravenous injection of [^{64}Cu]CuCl₂ and [^{18}F]FDG, respectively.

Table 1

	[^{64}Cu]CuCl ₂ 0.5 h p.i.	[^{64}Cu]CuCl ₂ 2 h p.i.	[^{64}Cu]CuCl ₂ 24 h p.i.	[^{18}F]FDG 0.5 h p.i.	[^{18}F]FDG 2 h p.i.
Liver	11.8 ± 7.4 *	16.6 ± 5.4	16.74 ± 6.3	4.73 ± 1.02	4.43 ± 1.12
heart	1.7 ± 1.3	1.96 ± 0.59	2.12 ± 0.62	29.4 ± 14.0	30.9 ± 11.9
Muscle	0.51 ± 0.37	0.59 ± 0.17	0.66 ± 0.31	0.29 ± 0.35	1.16 ± 0.31
brain	0.57 ± 0.41	0.58 ± 0.17	0.80 ± 0.34	7.13 ± 2.02	5.62 ± 1.92
Kidney	5.7 ± 4.0	6.14 ± 1.37	8.28 ± 3.26	11.9 ± 1.74	4.65 ± 1.71
Lung	2.1 ± 1.6	2.32 ± 0.70	2.26 ± 0.69	2.48 ± 1.65	1.58 ± 0.92

* %ID/g, percentage of injected dose per gram, mean ± standard deviation (SD)

** , h, hour; p.i., post intravenous injection.

Biodistribution of [^{64}Cu] CuCl_2 and [^{18}F]FDG (% ID/g) in the brain of Balb/c mice by PET/CT after intravenous injection of the tracers.

Table 2

	[^{64}Cu] CuCl_2 0.5 h p.i.	[^{64}Cu] CuCl_2 2 h p.i.	[^{64}Cu] CuCl_2 24 h p.i.	[^{18}F]FDG 0.5 h p.i.	[^{18}F]FDG 2 h p.i.
ROI 1 OB	0.36 $\pm$ 0.31	0.79 $\pm$ 0.21	0.89 $\pm$ 0.62	6.5 $\pm$ 1.7	6.3 $\pm$ 1.0
ROI 2 FC	0.39 $\pm$ 0.31	0.59 $\pm$ 0.18	0.47 $\pm$ 0.23	5.8 $\pm$ 1.7	4.9 $\pm$ 1.1
ROI 3 PC	0.31 $\pm$ 0.21	0.52 $\pm$ 0.17	0.41 $\pm$ 0.17	6.1 $\pm$ 2.0	4.8 $\pm$ 1.3
ROI 4 HC	0.64 $\pm$ 0.50	0.63 $\pm$ 0.28	0.82 $\pm$ 0.24	7.8 $\pm$ 2.4	5.7 $\pm$ 2.0
ROI 5 BG	0.67 $\pm$ 0.59	0.64 $\pm$ 0.15	0.92 $\pm$ 0.24	6.9 $\pm$ 2.2	6.1 $\pm$ 1.8
ROI 6 T	0.63 $\pm$ 0.49	0.60 $\pm$ 0.12	0.85 $\pm$ 0.39	7.6 $\pm$ 2.3	5.5 $\pm$ 1.9
ROI 7 MB	0.50 $\pm$ 0.38	0.45 $\pm$ 0.14	0.77 $\pm$ 0.22	9.2 $\pm$ 3.0	5.9 $\pm$ 2.0
ROI 8 BS	0.86 $\pm$ 0.68	0.77 $\pm$ 0.14	1.05 $\pm$ 0.27	8.2 $\pm$ 2.6	6.3 $\pm$ 2.4
ROI 9 CB	0.50 $\pm$ 0.41	0.64 $\pm$ 0.26	0.64 $\pm$ 0.13	8.5 $\pm$ 2.5	5.8 $\pm$ 2.1

* % ID/g, percentage of injected dose per gram, mean $\pm$ standard deviation (SD)

** ROI, region of interest; h, hour; p.i., post intravenous injection.

Table 3

^{64}Cu uptake (%ID/g) in the brains of *Atp7b*^{-/-} and C58BL/6 mice by PET/CT after intravenous injection of [^{64}Cu]CuCl₂ as a tracer.

	<i>Atp7b</i> ^{-/-} 2 h p.i.	<i>Atp7b</i> ^{-/-} 24 h p.i.	C57BL/6 2 h p.i.	C57BL/6 24 h p.i.
ROI 1 (OB)	0.53 ± 0.18	0.19 ± 0.08	0.49 ± 0.11	0.61 ± 0.19
ROI 2 (FC)	0.39 ± 0.10	0.11 ± 0.07	0.45 ± 0.12	0.50 ± 0.16
ROI 3 (PC)	0.36 ± 0.11	0.07 ± 0.04	0.48 ± 0.09	0.41 ± 0.11
ROI 4 (HC)	0.45 ± 0.16	0.21 ± 0.16	0.54 ± 0.15	0.83 ± 0.17
ROI 5 (BG)	0.66 ± 0.23	0.40 ± 0.17	0.50 ± 0.08	0.70 ± 0.20
ROI 6 (T)	0.41 ± 0.17	0.33 ± 0.03	0.48 ± 0.04	0.57 ± 0.17
ROI 7 (MB)	0.33 ± 0.06	0.24 ± 0.12	0.30 ± 0.07	0.51 ± 0.11
ROI 8 (BS)	0.33 ± 0.11	0.32 ± 0.08	0.63 ± 0.21	0.70 ± 0.11
ROI 9 (CB)	0.43 ± 0.14	0.15 ± 0.18	0.45 ± 0.12	0.57 ± 0.05

* %ID/g, percentage of injected dose per gram, mean ± standard deviation (SD)

** ROI, region of interest; h, hour; p.i., post intravenous injection.

Age-dependent changes of ^{64}Cu uptake (%ID/g) in the brains of *Atp7b*^{-/-} mice by longitudinal PET/CT at 24 h after oral administration of [^{64}Cu]CuCl₂ as a tracer

Table 4

	<i>Atp7b</i> ^{-/-} 3 th week	<i>Atp7b</i> ^{-/-} 18 th week	<i>Atp7b</i> ^{-/-} 21 th week	<i>C57BL/6</i> 13 th week	<i>C57BL/6</i> 18 th week	<i>C57BL/6</i> 21 th week
ROI 1 OB	0.12 ± 0.14	0.23 ± 0.18	0.30 ± 0.20	0.30 ± 0.12	0.15 ± 0.04	0.15 ± 0.05
ROI 2 FC	0.04 ± 0.04	0.20 ± 0.06	0.23 ± 0.03	0.22 ± 0.01	0.12 ± 0.06	0.06 ± 0.05
ROI 3 PC	0.04 ± 0.03	0.14 ± 0.09	0.14 ± 0.00	0.23 ± 0.04	0.12 ± 0.05	0.04 ± 0.03
ROI 4 HC	0.04 ± 0.03	0.15 ± 0.02	0.15 ± 0.10	0.23 ± 0.11	0.18 ± 0.11	0.12 ± 0.07
ROI 5 BG	0.06 ± 0.04	0.21 ± 0.05	0.20 ± 0.03	0.16 ± 0.04	0.17 ± 0.06	0.16 ± 0.07
ROI 6 T	0.05 ± 0.03	0.12 ± 0.04	0.19 ± 0.05	0.22 ± 0.07	0.18 ± 0.05	0.13 ± 0.06
ROI 7 MB	0.05 ± 0.03	0.11 ± 0.01	0.18 ± 0.07	0.23 ± 0.11	0.15 ± 0.07	0.09 ± 0.03
ROI 8 BS	0.05 ± 0.04	0.09 ± 0.06	0.16 ± 0.08	0.24 ± 0.05	0.23 ± 0.06	0.12 ± 0.04
ROI 9 CB	0.07 ± 0.07	0.11 ± 0.04	0.36 ± 0.11	0.11 ± 0.05	0.20 ± 0.06	0.09 ± 0.05

* % ID/g, percentage of injected dose per gram, mean ± standard deviation (SD)

** ROI, region of interest; h, hour.