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ORIGINAL ARTICLE

Association of copper levels in the hair with gray matter volume, mean diffusivity, and cognitive functions

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Abstract

Although copper plays a critical role in normal brain functions and development, it is known that excess copper causes toxicity. Here we investigated the associations of copper levels in the hair with regional gray matter volume (rGMV), mean diffusivity (MD), and cognitive differences in a study cohort of 924 healthy young adults. Our findings showed that high copper levels were associated mostly with low cognitive abilities (low scores on the intelligence test consisting of complex speed tasks, involving reasoning task, a complex arithmetic task, and a reading comprehension task) as well as lower reverse Stroop interference, high rGMV over widespread areas of the brain [mainly including the bilateral lateral and medial parietal cortices, medial temporal structures (amygdala, hippocampus, and parahippocampal gyrus), middle cingulate cortex, orbitofrontal cortex, insula, perisylvian areas, inferior temporal lobe, temporal pole, occipital lobes, and supplementary motor area], as well as high MD of the right substantia nigra and bilateral hippocampus, which are indicative of low density in brain tissues. These results suggest that copper levels are associated with mostly aberrant cognitive functions, greater rGMV in extensive areas, greater MD (which are indicative of low density in brain tissues) in subcortical structures in the healthy young adults, possibly reflecting copper's complex roles in neural mechanisms.

Keywords Copper · Regional gray matter volume · Mean diffusivity · Cognitive functions

Introduction

Copper (Cu) is an essential trace element that plays an important role in humans. Copper serves as a cofactor for several enzymes, such as cytochrome oxidase, CuZu-superoxide dismutase, lysine oxidase, dopamine-β-hydroxylase, and ceruloplasmin (Paris et al. 2001). On the one hand, Cu is essential for optimal antioxidant defense, and Cu deficiency inhibits the body's ability to deal with oxidative stress through decreased capacity of producing the antioxidant superoxide dismutase (Gaetke and Chow 2003). On the other hand, in the brain, copper plays an important role in the production of neurotransmitters, and the enzyme that converts dopamine into norepinephrine is dependent on Cu (Friedman and Kaufman 1965). Further, animals with dietary copper deficiency exhibit reduced noradrenalin and dopamine concentrations (Hunt 1980).

Ceruloplasmin catalyzes the oxidation of the biogenic amines noradrenaline and serotonin (Frieden 1980). Cu is required for brain development, and mammals with Cu deficiency during development exhibit smaller brains (Everson et al. 1967) and reduced synthesis of brain tissues (Krejpcio et al. 1997). Further, even a very small Cu deficiency disturbs the maturation of the hippocampus and dentate gyrus (Hunt and Idso 1995), and copper protects against neuronal death caused by abnormal hyperactivity (Hashimoto 2008).

In contrast, Cu is a redox-active transition metal, excessive Cu is supposed to initiate oxidative damage, and high Cu concentrations may cause increased oxidative damage to lipids, proteins, and DNA and contribute to neurodegenerative disorders (Gaetke and Chow 2003). In particular,

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Cu neurotoxicity has been investigated, and Cu has been suggested to play an important role in neurodegeneration (Paris et al. 2001). A previous study has suggested that Cu neurotoxicity is partly dependent on the reaction between dopamine and Cu, including the formation of Cu–dopamine complexes with concomitant dopamine oxidation, which generates the substance responsible for the degeneration of dopaminergic neurons in the substantia nigra (Segura-Aguilar et al. 2001) where Cu accumulates with aging (Zecca et al. 2005). Further, the association between neurotoxicity and copper has attracted attention, particularly in relation to Alzheimer's disease, and numerous animal, human experimental, clinical, and observational studies have been devoted to this issue (for reviews, refer to Brewer 2009, 2012; Loef and Walach 2012; Squitti et al. 2014a; Pal et al. 2015). Among such studies, an animal study has shown that among rabbits with high cholesterol levels, a small amount of copper leads to learning disability and accumulation of amyloid beta in the hippocampus and temporal lobe (Hashimoto 2008).

Previous human physiological studies have found that greater Cu levels are associated with lower testosterone levels (Chang et al. 2011). Moreover, high Cu levels in the body are associated with learning disabilities, juvenile delinquency, schizophrenia, and autism (Rimland and Larson 1983; Rahman et al. 2009; Priya and Geetha 2011). Further, among studies relevant to Alzheimer's disease, a large epidemiological study has found that among humans with high blood cholesterol levels, high Cu levels are associated with accelerated age-related cognitive decline (Morris et al. 2006). The body copper level is inversely related to lower cognitive function in older adults (Salustri et al. 2010), and patients with Alzheimer's disease show elevated hair copper levels (Koc et al. 2015) as well as greater free copper levels in blood (Squitti et al. 2014b). Further, a greater serum free copper level is associated with an unfavorable prognosis of cognitive function in patients with Alzheimer's disease (Squitti et al. 2009). A neuroimaging study has found that childhood exposure to lead (a mineral known for its neurotoxicity) leads to decreased regional gray matter volume rGMV in widespread regions of the brain (Cecil et al. 2008).

Despite these previous studies, the following points have not been investigated:

(a) The associations between Cu levels in the body and cognitive functions among a large cohort of young adults.
(b) The associations between Cu levels in the body and brain structural properties among a large cohort of young adults.

The purpose of this study was to investigate these issues. For this purpose, we utilized a wide range of cognitive tests, regional gray matter volume (rGMV) and mean diffusivity (MD), and determined hair Cu levels in a relatively large cohort of young adults. rGMV and MD are both frequently assessed in the investigations of the long-term effects of environmental factors such as the effects of childhood exposure to environmental factors, including metals, in later life (Cecil et al. 2008; for review of cases of rGMV, see; Takeuchi and Kawashima 2016; Takeuchi et al. 2016a).

As described above, despite Cu's critical role in neurophysiology and development, an accumulated body of evidence has suggested that unless there is a deficit in Cu levels, an elevated Cu level is associated with aberrant neurocognitive conditions. Thus, we hypothesized that among normal young adults in developed countries, high Cu levels are associated with (a) diminished cognitive functions such as reading and arithmetic abilities due to the abovementioned associations of greater copper levels and learning disabilities (Rimland and Larson 1983). Also, excess Cu may be associated with (a) even basic cognitive abilities such as working memory capacity and executive function, consistent with the abovementioned associations of high copper levels with age-related cognitive decline, psychiatric diseases, and developmental disorders (Morris et al. 2006; Rahman et al. 2009; Priya and Geetha 2011); (b) reduced cognitive abilities, traits and states related to dopaminergic functions, such as creativity measured by divergent thinking, extraversion, novelty seeking, and state of vigor (for summary, see Takeuchi et al. 2013c); and (c) aberrant brain structure, such as lower rGMV and greater MD (indicative of lower tissue density) (Johansen-Berg et al. 2012), particularly in the substantia nigra and hippocampus.

Methods

Subjects

The present study, which is a part of an ongoing project to investigate the association between brain imaging, cognitive function, and aging, included 924 healthy, right-handed individuals (563 men and 361 women) from whom the data necessary for whole brain analyses involving Cu levels were collected. The mean age of the subjects was 20.7 years [standard deviation (SD), 1.8; age range: 18–27 years old]. All subjects were university students, postgraduates, or university graduates of less than 1 year's standing. All subjects had normal vision and none had neurological or psychiatric illness, and this has been confirmed by a self-report. Therefore, we do not consider our sample includes typically neurologically pathological sample. In this study, we recruited subjects who were not taking drugs without specifying details of types of drugs through ads. But still sometimes, some subjects who were taking drugs applied and during

the selection process after application, whereas subjects who were taking drugs for brain diseases were excluded, subjects who were taking drugs used in everyday lives such as the drugs for cold, allergy, and contraceptives in the self-report were not removed. Handedness was evaluated using the Edinburgh Handedness Inventory (Oldfield 1971). For details of subjects' information, see "Supplemental Methods". For the limitation of this study related to subjects' characteristics, see "Supplemental Discussion".

Hair acquisition and hair mineral analysis

As summarized in previous studies (Priya and Geetha 2011; Takeuchi et al. 2013c), hair mineral analysis serves as the best indicator of mineral levels in the body and has gathered considerable interest in multiple fields (Lech 2002). Hair is recognized as a potential repository of all elements that enter the body, and mineral levels in the hair indicate the mineral composition accumulated over several months to years (Priya and Geetha 2011). Correlations exist between the concentrations of basic elements in the hair and in the body (Chłopicka et al. 1998; Kedzierska 2003). Hair mineral analysis has advantages over other methods, such as blood and urine analyses, in investigating mineral levels in the body because mineral levels in the hair are not subjected to rapid fluctuations of mineral intake and have long-term stability (Ayodele and Bayero 2009).

Scalp hair samples (approximately 4-cm length, 0.1-g weight) were collected from each subject, with the hair cut as close to the scalp as possible. The hair samples were sent to La Belle Vie research laboratory and analyzed by established methods, as described previously (Takeuchi et al. 2013c). The length of the hair sample was prescribed by the company and it is a standard procedure. For details, see Supplemental Methods.

There is continuing debate regarding whether hair or blood sampling is more appropriate for investigation of the effects of body copper levels (Wilson 2003). New analytic methods and good practice have improved the precision of hair mineral analysis (Bass et al. 2001). Well controlled changes in copper intake substantially changed hair copper level but not serum copper level in humans (Turnlund et al. 2004). Through experiments on pigs, hair copper was substantially increased when the dietary copper level was increased (Castell and Bowland 1968); hair copper concentration has been found to reflect liver copper concentration in pigs (Jacob et al. 1978). A previous study robustly showed that hair copper levels of children in the city near copper smelter where the levels in water and snow far exceeded existing standards were substantially greater than those of children in the control cities, whereas this effect was not observed for blood copper levels. A previous study showed that 24-h urine copper output following oral chelation with D Penicillamine was strongly correlated with hair copper level, suggesting the validity of "hair mineral analysis" as an accurate monitor of the total body copper load (Nolan 1983). Blood and tissue testing are different and provide valuable information. Other than practical easiness for sampling, hair mineral analyses have the following advantages in measuring long-term body copper loading when properly measured: Hair sampling provides long-term copper loading that is unaffected by daily events and is suitable for the investigation of long-term effects of the copper level (Wilson 2003). Serum mineral levels are maintained at the expense of the tissues even in serious illnesses (Wilson 2003). Excess minerals, including toxic metals, are quickly removed from blood and deposited in tissues including hair (Wilson 2003).

Psychological measures

Following neuropsychological tests and questionnaires were administered. These tests are described in this subsection and were largely reproduced from our previous studies (Takeuchi et al. 2013a, 2015d). [A] A (computerized) digit span task, which is a working memory task (for details, see Takeuchi et al. 2011b). [B] RAPM (Raven 1998), a non-verbal reasoning task and representative measure of general intelligence. For more details, see our previous study (Takeuchi et al. 2010). [C] Tanaka B-type intelligence test (Tanaka et al. 2003) type 3B (TBIT). This non-verbal mass intelligence test, used for 3rd-year junior high school and older examinees, does not include story problems but uses figures, single numbers, and letters as stimuli. In all subtests, the subjects have to solve as many problems as possible before a certain time (a few minutes), meaning these are complex cognitive speed tasks. For more details, see our previous study (Takeuchi et al. 2013a). There are three subfactors, namely the perception factor, the spatial relation factor, and the reasoning factor. The perception factor measures simple processing speed. The spatial relation factor measures spatial abilities to relate different things. The reasoning factor of TBIT measures reasoning abilities. [D] Arithmetic tasks. These tests measure multiplication performance consisting of two forms of one-digit times one-digit multiplication problems (a simple arithmetic task with numbers between 2 and 9) and two forms of two-digit times two-digit multiplication problems (a complex arithmetic task with numbers between 11 and 19). The two forms of each task are same, but the numbers used in the problems are ordered differently. Each form of the simple and complex arithmetic tasks has to be completed in 30 and 60 s, respectively. [E] The Stroop task (Hakoda's version) (Hakoda and Sasaki 1990), which measures response inhibition and impulsivity. Hakoda's version is a matching-type Stroop task requiring subjects

to check whether their chosen answers are correct, unlike the traditional oral naming Stroop task. The test consists of two control tasks (Word-Color and Color-Word tasks), a Stroop task, and a reverse Stroop task. Reverse Stroop and Stroop interference rates are calculated from these. See our previous study for details (Takeuchi et al. 2015c).

[F] Reading comprehension task. This task was developed by Kondo et al. (2003). For more details on this test, such as how it was developed and its validity, please refer to Kondo et al. (2003) and our previous study (Takeuchi et al. 2015d). [G] S-A creativity test. Creativity measured by divergent thinking was measured using the S-A creativity test (Society_For_Creative_Minds 1969). [H] The motivational status of the preceding 1 week for each subject was measured using the Vigor subscale of shortened Japanese version (Yokoyama 2005) of the Profile of Mood States psychological rating scale (McNair et al. 1992). [I] Novelty seeking score on a Japanese version (Kijima et al. 1996) of the Temperament Character Inventory (Cloninger et al. 1993), which was used to measure novelty seeking; [J] Extraversion scale on a Japanese version of the NEO Five-Factor Inventory (NEO-FFI) (Costa and McCrae 1992), which was used to measure extraversion.

Image acquisition

The methods for MR image acquisition were described in our previous study and reproduced below(Takeuchi et al. 2012). All MRI data acquisition was performed using a 3-T Philips Achieva scanner. High-resolution T1-weighted structural images (T1WIs: 240 × 240 matrix, TR = 6.5 ms, TE = 3 ms, FOV = 24 cm, slices = 162, slice thickness = 1.0 mm) were collected using a magnetization-prepared rapid gradient echo sequence.

Diffusion-weighted data were acquired using a spin-echo EPI sequence (TR = 10,293 ms, TE = 55 ms, FOV = 22.4 cm, 2 × 2 × 2 mm^3 voxels, 60 slices, SENSE reduction factor = 2, number of acquisitions = 1). The diffusion weighting was isotropically distributed along 32 directions (b value = 1000 s/mm^2). Additionally, three images with no diffusion weighting (b value = 0 s/mm^2) ($b = 0$ images) were acquired, using a spin-echo EPI sequence (TR = 10,293 ms, TE = 55 ms, FOV = 22.4 cm, 2 × 2 × 2 mm^3 voxels, 60 slices). FA and MD maps were calculated from the collected images using a commercially available diffusion tensor analysis package on the MR console. For more details, see "Supplemental Methods". Descriptions in this subsection were mostly reproduced from a previous study using similar methods (Takeuchi et al. 2016a). Quality of the all types of the obtained imaging data was checked by visual inspection and images of low quality were not used in this project.

Pre-processing of structural data

MD, measured using diffusion tensor imaging, is a direction-independent measure of how water molecules can freely move. MD is considered to reflect the amount of tissue in the brain, which prevents the free movement of water, and differences in MD may reflect tissue changes, such as astrocyte swelling, synaptic changes, dendritic spine changes, and angiogenesis; differences in MD are also sensitive to neural plasticity (Johansen-Berg et al. 2012; Sagi et al. 2012; Takeuchi et al. 2016a). MD in the dopaminergic system's areas (MDDS) is sensitive to the pathology of dopaminergic systems compared with other imaging modalities (Parkinson's disease) (Seppi et al. 2004; Péran et al. 2010). MDDS is sensitive to treatment with dopamine agonists used to manage this pathology (Razek et al. 2011) as well as to cognitive states and traits associated with the function of the dopaminergic system (Takeuchi et al. 2015a, 2016b).

Preprocessing of the structural data was performed using Statistical Parametric Mapping software (SPM12; Wellcome Department of Cognitive Neurology, London, UK) implemented in Matlab (Mathworks Inc., Natick, MA, USA). Using the new segmentation algorithm and the diffeomorphic anatomical registration through exponentiated Lie algebra (DARTEL) registration process implemented in SPM12, the T1-weighted structural images of each individual were segmented and normalized to the Montreal Neurological Institute (MNI) space to generate images with 1.5 × 1.5 × 1.5 mm^3 voxels. In addition, we performed a volume change correction (modulation) (Ashburner and Friston 2000). Subsequently, the generated rGMV images were smoothed by convolving them with an isotropic Gaussian kernel of 8 mm full width at half maximum (FWHM). For a full description of these procedures, see the "Supplemental Methods". The description in this paragraph was mostly reproduced from our previous study that used the same method (Takeuchi et al. 2017).

Preprocessing and analysis of imaging data were performed using SPM8 implemented in Matlab. Basically, we normalized MD images of subjects with previously validated diffeomorphic anatomical registration through exponentiated lie algebra (DARTEL)-based registration process method that utilizes the FA signal distribution within the white matter area in the normalization procedure to give images with 1.5 × 1.5 × 1.5 mm^3 voxels; then tissues that are not likely to be gray or white matter were carefully removed and smoothed by convolving them with an isotropic Gaussian kernel of 8-mm full width at half maximum. For details, see "Supplemental Methods". The description in this subsection was mostly reproduced from our previous study that used the same method (Takeuchi et al. 2016b).

This method can remove partial volume effects of CSF through thorough removal of CSF areas and achieve accurate

normalization within the white matter areas by utilizing the FA signal distribution within the white matter area in the normalization procedure. The congruence of findings obtained by tract-based spatial statistics and those obtained by our preprocessing method was shown in our previous study (Takeuchi et al. 2013b). One of the strengths of our preprocessing method is the ability to obtain MD signals in gray matter areas, particularly subcortical regions, which are known to be important in a wide range of studies (Takeuchi and Kawashima, in press).

Whole-brain statistical analysis

We investigated if the rGMV was associated with individual differences in the hair copper level. The statistical analyses of imaging data were performed with SPM8. In these analyses, we performed a whole-brain multiple regression analysis. These analyses were performed with sex, age, and total intracranial volume (TIV) that was calculated as described previously (Hashimoto et al. 2015), self-reported height, self-reported weight, and body mass index (BMI), which was calculated from the self-reported height and self-reported weight and Cu levels in the hair. We included only voxels with an rGMV signal intensity of >0.05 for all participants. We used SPM12 for VBM preprocessing and SPM8 for statistical analyses because of the compatibility of the software that we used for permutation-based statistics described below and of the script we used for the statistical analyses. If permutation tests were used, the results were not affected by the version of SPM.

The investigation of the associations between regional MD and Cu levels in the hair was performed using a whole-brain multiple regression analysis. The statistical model was same as that of rGMV, except that the whole-brain multiple regression analysis for MD did not include TIV as a covariate. The analyses were limited to the gray + white matter mask, which was created as described above.

A multiple comparison correction was performed using threshold-free cluster enhancement (TFCE) (Smith and Nichols 2009) with randomized (5,000 permutations) non-parametric testing using the TFCE toolbox (http://dbm.neuro.uni-jena.de/tfce/). We applied a threshold of FWE corrected at $P<0.05$.

Regions of interest (ROI) analyses of the associations between Cu levels of the hair and MD

We employed ROI approaches to determine the MD of the bilateral substantia nigra and of the bilateral hippocampus and Cu levels in the hair. The reasons for focusing on these anatomical areas are described in the "Introduction" section. The mean MD values of the left and right substantia nigra and left and right hippocampus were extracted. For details, see "Supplemental Methods".

Statistical analyses of non-whole brain analyses

The associations were tested using multiple regression analyses. The dependent variable was the mean MD in one of the ROIs or cognitive variables that were cited in the subsection "Psychological measures". Independent variables comprised sex, age, self-reported height, self-reported weight, and BMI that was calculated from the self-reported height and self-reported weight, and Cu levels in the hair.

In these analyses, results with a threshold of $P<0.05$ were considered to be statistically significant after correcting for the false discovery rate (FDR) using the graphically sharpened method (Benjamini and Hochberg 2000). This correction for multiple comparisons was performed among 19 non-whole brain multiple regression analyses (15 dependent variables of psychological measures and 4 dependent variables of MD).

Although the linear regression analyses showed mostly significant results, from the information presented in the Introduction, one might expect a nonlinear relationship. According to our previous study (Taki et al. 2011), to analyze whether the linear or quadratic function best fits the trajectory of the mean MD in one of the ROIs or cognitive variables that were cited in the subsection of Psychological measures with Cu levels in the hair, the correlations of these associations were estimated using linear and quadratic functions in each ROI. However, these results did not achieve the best possible fit for quadratic functions in an expected way. Further, these analyses must be performed without covariates that serve as a reference. Therefore, we only reported the results of multiple regression analyses showing significant results.

Further, it is known both Cu and iron have similar binding proteins in cerebral tissue and they affect each other during uptake (Fox 2003; Ha et al. 2018); therefore, the iron level might affect the present results. To exclude such possibilities, we additionally performed non-whole brain multiple regression analyses including hair iron level as an additional covariate. Here to avoid double dipping procedures in whole brain multiple regression analyses, for GMV analyses, we used total GMV as a dependent variable. We confirmed that the addition of hair iron level as a covariate did not affect substantially influence the strengths of significant associations. For these methods and results, see "Supplemental Methods", "Supplemental Results", and "Supplemental Table 1".

We did not perform mediation analyses to see if neural mechanisms mediate the associations between hair copper levels and cognitive functions and between hair copper levels and neural mechanisms. We present the correlations

among hair copper levels and significant psychological and neural correlates of hair copper levels after correcting for confounding variables in Supplemental Table 2 (rGMV of the large cluster of significant correlations was replaced by total GMV to avoid double-dippingprocedures) (Kriegeskorte et al. 2009) Since hair copper levels mostly negatively correlated with cognitive functions as presented in "Results", if neural mechanisms mediate the associations of hair copper levels with cognitive functions, neural mechanisms should correlate with hair copper levels and cognitive functions in opposite ways. Mostly, there were no such significant correlations, although there is one such correlation, and total GMV negatively correlated with complex calculation performance and positively correlated with hair copper level; p-value was only marginally significant and among many statistical tests, it is difficult to gage the significance of the findings. This could be due to the potentially complex mechanisms of the associations of copper levels with neural mechanisms (see the third paragraph of "Discussion").

Results

Basic data

The mean and standard deviation for age, general intelligence test score, and hair Cu levels (raw and log) are presented in Table 1. Average (± SD) raw hair copper level was 24.01 (± 31.28) μg/g in males and 27.34 (± 18.72) μg/g in females. In most of the studies on normal subjects, the average hair copper levels ranged from 10 to 35 or 40 μg/g; therefore, our samples fall within a normal range (Sakai 1970), and this average value is well within the reference range for the hair copper levels of normal subjects reported in a previous review (Mikulewicz et al. 2013) and similar to the value (22.4 μg/g) for young Japanese adults measured by the same company (La Belle Vie Inc, Tokyo, Japan). The distribution of the logarithms of Cu levels in the hair for men and women are presented in Supplemental Fig. 1.

Table 1 The demographics of study participants

Measure	Male ($N=563$)		Female ($N=361$)	
	Mean	SD	Mean	SD
Age	20.83	1.93	20.54	1.64
RAPM	28.77	3.89	28.00	3.87
Log-Copper	4.31	0.2	4.37	0.22
Raw hair copper level (μg/g)	24.01	31.28	27.34	18.72

Psychological analyses of the correlations between Cu levels in the hair and individual cognitive differences

Psychological analyses revealed that after correcting for confounding variables and multiple comparisons, Cu levels in the hair significantly and negatively correlated with the total intelligence score of TBIT, the reasoning factor of TBIT, complex arithmetic, reverse Stroop interference, and a reading comprehension task, but not with the other cognitive functions, traits, and states that are related to dopaminergic functions (Fig. 1). The results of all statistical analyses are presented in Table 2.

Analyses of the correlations between Cu levels in the hair and rGMV

The whole-brain multiple regression analysis showed that Cu levels in the hair significantly and positively correlated with rGMV in widespread areas, mainly including the bilateral lateral and medial parietal cortices, medial temporal structures (amygdala, hippocampus, and parahippocampal gyrus), middle cingulate cortex, orbitofrontal cortex, insula, perisylvian areas, inferior temporal lobe, temporal pole, occipital lobes, and supplementary motor area (Fig. 2; Table 3).

Analyses of the correlations between Cu levels in the hair and MD

The whole-brain multiple regression analysis showed that Cu levels in the hair did not significantly correlate with MD in any of the brain regions. ROI analyses of the bilateral substantia nigra and bilateral hippocampus showed that Cu levels in the hair positively and significantly correlated with the MD of the right substantia nigra (Fig. 3; Table 4; $\beta=0.082$, $t=2.624$, $p=0.009$), MD of the right hippocampus (Fig. 3; Table 4; $\beta=0.069$, $t=2.127$, $p=0.034$), and MD of the left hippocampus (Fig. 3; Table 4; $\beta=0.068$, $t=2.131$, $p=0.034$), but not with the MD of the left substantia nigra (Fig. 3; Table 4; $\beta=-0.010$, $t=-0.303$, $p=0.762$).

Discussion

In the present study, the associations of Cu levels in the hair with a wide range of cognitive differences, rGMV, and MD were investigated among a relatively large cohort of young adult subjects in a developed country. Partially consistent with our hypothesis, our novel findings showed that high Cu levels were associated with a low total intelligence score of tests comprising time tasks, reasoning factors of this test, complex arithmetic tasks, and a reading comprehension task. However, partially inconsistent with our hypothesis,

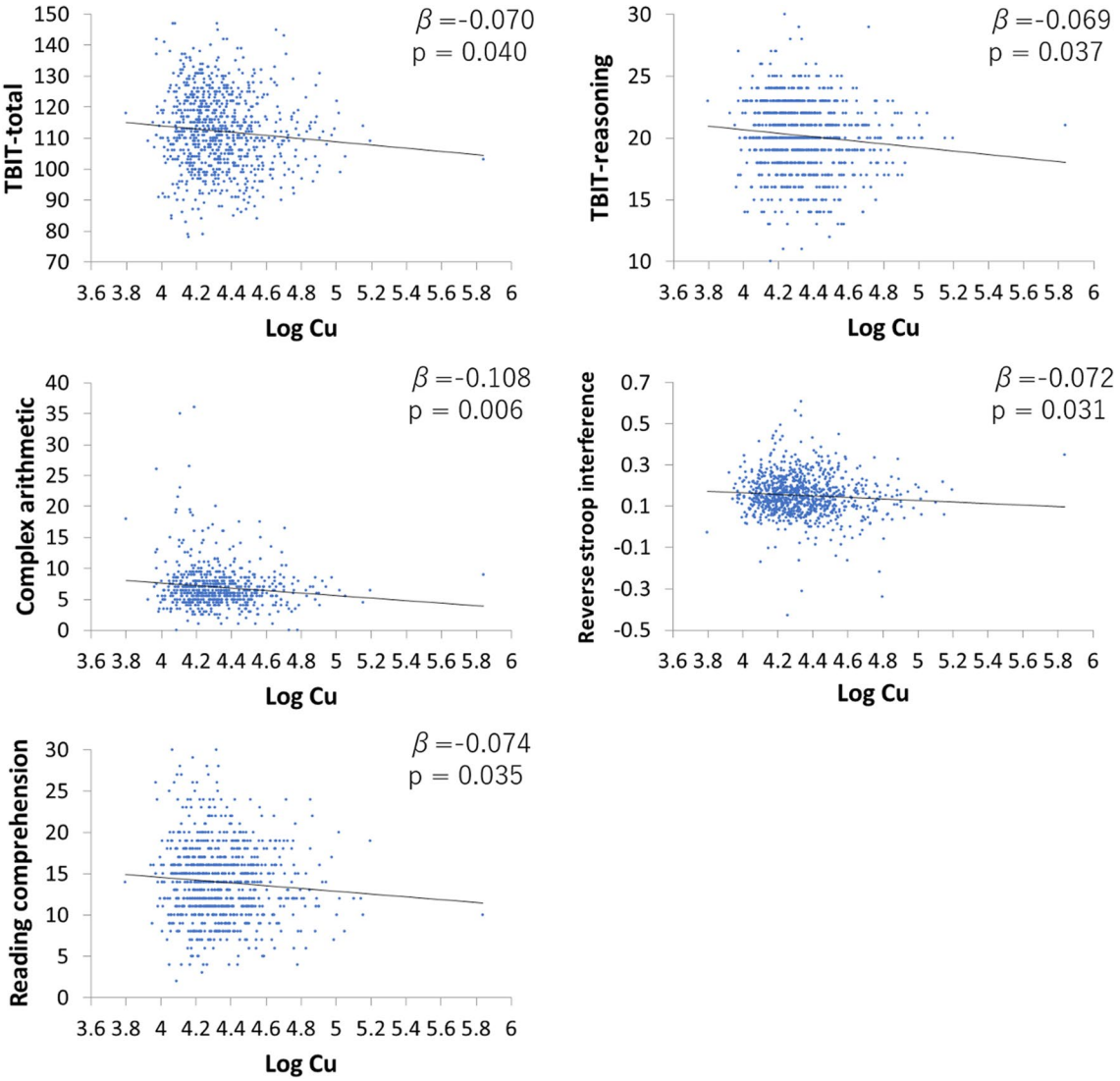

Fig. 1 Scatter plots of significant associations between copper (Cu) levels in the hair (logarithms of copper levels in the hair) and cognitive functions. The scatter plots include associations of the Cu level in the hair with the total intelligence score of TBIT, reasoning factor of TBIT, complex arithmetic, reverse Stroop interference, and a reading comprehension task. The statistical values were those of the multiple regression analyses after correcting for confounding variables

high Cu levels were associated with reduced reverse Stroop interference (indicator of the ability to resolve cognitive interference). Further, partially consistent with our hypothesis, high Cu levels were associated with high MD of the right substantia nigra and bilateral hippocampus, which were indicative of low densities of various neural tissues in the regions described below. However, partially inconsistent with our hypothesis, high Cu levels were associated with high rGMVs in widespread areas, mainly including the bilateral lateral and medial parietal cortices, medial temporal structures (including the hippocampus), middle cingulate cortex, orbitofrontal cortex, insula, perisylvian regions, inferior temporal lobe, temporal pole, occipital lobes, and supplementary motor areas. Finally, inconsistent with our hypothesis, Cu levels did not show significant relationships with cognitive abilities, traits, and states associated with dopaminergic functioning.

In this study, there are few possible limitations and possible confounding factors that may be considered before discussing specific results. (1) We believe it is unlikely that the present imaging results were caused by the magnetic properties of Cu in the brain. For example, the physiological Cu concentrations in the brain are too small to produce a detectable MR contrast (Bai et al. 2013). Further, Cu has a paramagnetic property, and the accumulation of paramagnetic minerals may lead to decreased signals in MD images and related images (Chandarana et al. 2012). However, the present study showed a high MD signal in subjects with

Table 2 Statistical results (beta value, *t* value, uncorrected *p* values, *p* value corrected for FDR) for the multiple regression analyses performed using psychological variables and the copper level after correcting for confounding variables

Dependent variables	*N*	*β*	*t*	Cu level	
				p (uncorrected)	*p* (FDR)
RAPM[a]	919	− 0.044	− 1.326	0.185	0.107
Total intelligence score of TBIT[b]	847	− 0.070	− 2.060	0.040	0.040
Perception score of TBIT	847	− 0.066	− 1.884	0.060	0.053
Spatial relation factor of TBIT	853	− 0.036	− 1.068	0.286	0.152
Reasoning factor of TBIT	853	− 0.069	− 2.085	0.037	0.040
Simple arithmetic	664	− 0.030	− 0.775	0.439	0.219
Complex arithmetic	664	− 0.108	− 2.767	0.006	0.035
Reverse stroop interference	922	− 0.072	− 2.164	0.031	0.040
Stroop interference	921	0.044	1.318	0.188	0.107
Reading comprehension	840	− 0.074	− 2.114	0.035	0.040
S-A creativity test	924	− 0.049	− 1.483	0.138	0.092
Digit span	919	− 0.009	− 0.268	0.789	0.332
POMS[c]-Vigor	913	− 0.057	− 1.718	0.086	0.069
Extraversion	923	− 0.023	− 0.684	0.494	0.232
Novelty seeking	922	0.053	1.591	0.112	0.081

The table presents the beta values, *t* values, uncorrected *p* values, and *p* values corrected for FDR[a] for the multiple regression analyses performed using psychological variables and Cu levels after correcting for confounding variables

FDR false discovery rate

[a]Raven's advanced progressive matrices (a general intelligence task)

[b]Tanaka B-type intelligence test

[c]Profiles of Mood States

high Cu levels in the hair. (2) The present cohort comprised educated people who were young and healthy. which is a common caveat of studies on college students (Jung et al. 2010) in developed countries. Among subjects with symptoms of Cu deficiency and subjects with high cholesterol levels, where higher copper intake is associated with cognitive decline (Morris et al. 2006), how Cu levels associate with cognitive and neural variables may differ in such samples. Future studies need to investigate these issues. (3) Further, Cu is involved in neurotransmitter synthesis and to assess the relevant associations of such physiological properties (Paris et al. 2001), the measures that can specifically tap these acute changes, such as blood Cu level, and dopamine synthesis capacity by PET imaging, might be more appropriate. Future studies need to address these issues. (4) Further, although copper or ceruloplasmin level in blood is affected by estrogen, the estrus cycle (Pfeiffer and Mailloux 1987), and contraceptives (Vir and Love 1981), we did not consider these factors. Our procedure is common to most of the relevant studies involving brain structural studies and hair mineral analyses. As for the estrus cycle, in this study, we used a hair copper level from a 4-cm hair sample that corresponded to hair of a few months as an independent variable of interest. The 4-cm hair sample would reflect the temporal average Cu metabolism over several unsynchronized estrus cycles; therefore, we believe that the estrus cycle is unlikely to be confounding variable. As for contraceptives, the additional supplemental analyses revealed that the removal of the subjects taking contraceptives did not alter the statistical strength of the significant results in the main text substantially, and we believe that the effects on the present findings are negligible (see "Supplemental Methods", "Supplemental Results" and "Supplemental Table 3"). We also conducted analyses for sex-specific effects but found no significant interaction between sex and hair copper levels on significant correlates of the hair copper levels that were presented in the main text (see "Supplemental Methods", "Supplemental Results" and "Supplemental Table 4"). (5) Finally, we were unable to determine the causal relationship or micro-level mechanisms of the observed associations between Cu levels, rGMV, MD, and cognitive functions from macro-level cross-sectional neuroimaging studies. However, for reference, we discussed possible mechanisms in the following discussion.

In the present study, although Cu levels were associated with high MD and diminished cognitive functions, they were associated with high rGMVs in widespread regions in the brain. Sufficient Cu levels are critical to the normal development of the brain (Hunt and Idso 1995), and animal experiments have shown that even a small Cu deficiency disturbs the maturation of the hippocampus (Hunt and Idso 1995). A previous review of the effects of environmental factors on rGMV has suggested that extrinsic factors (e.g.,

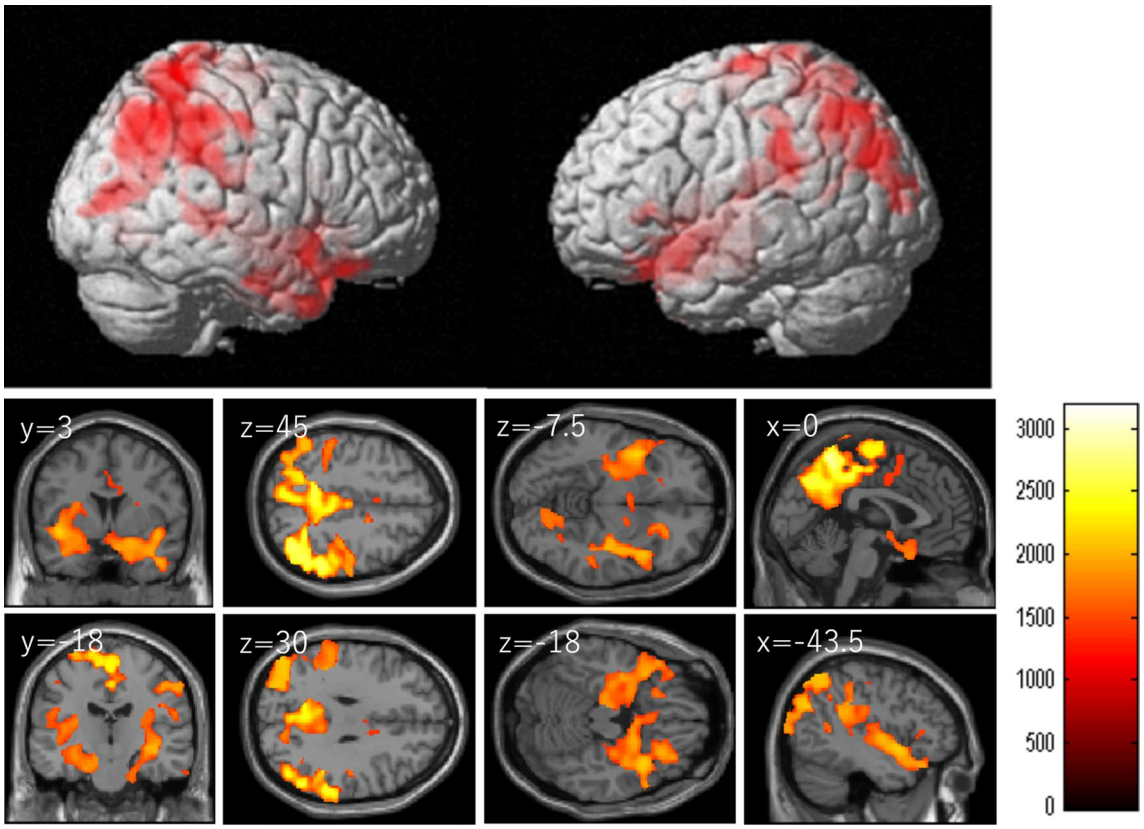

Fig. 2 The positive rGMV correlates with Cu levels in the hair. The results were obtained using a threshold of threshold-free cluster enhancement (TFCE) of $P < 0.05$ based on 5,000 permutations. The results were corrected at the whole-brain level. Upper panel: regions with significant correlations are projected on a rendered SPM8 image. Significant positive correlations with rGMV were observed in extensive cortical regions, particularly in the parietal lobes and in regions of perisylvian areas. Lower panels: regions with significant correlations between rGMV and Cu levels in the hair are overlaid on a "single subject" T1 image from SPM8. Significant positive correlations between rGMV and Cu levels in the hair were observed in extensive regions throughout the brain, particularly in the lateral and medial parietal lobes, perisylvian areas, medial temporal areas, and the insula. The color bar represents the TFCE score. It reflects both voxel's height and the sum of the spatially contiguous voxels supporting it; therefore, it reflects both the strength and extent of effects

nutrition and toxins) tend to globally affect rGMV to a significant extent during an earlier stage of brain development (Takeuchi and Kawashima 2016). Perhaps the contribution of high Cu levels to the normal development of the brain at an early stage is more dominant than other nonadaptive effects of high Cu levels. Partially consistent with these results, it was suggested that low Cu concentrations may result in incomplete development, whereas excess Cu concentrations may be injurious to the brain (Desai and Kaler 2008). Further, studies have suggested that during development, cortical thinning that is supposed to reflect adaptive synaptic pruning occurs, and evidence has suggested that steep cortical thinning during development is associated with increased psychometric intelligence (Shaw et al. 2006). Thus, although we are not aware of the studies indicating that Cu disturbs synaptic pruning, other mechanisms are possible.

The present study does not identify the mechanism of regional specificity of the associations between Cu levels in the hair and rGMV. The associations between Cu levels and rGMV were detected in certain areas, including the hippocampus, but not in other regions. However, the absence of a significant association in other areas is not the evidence of lack and this may be attributed to the insufficient statistical power required to detect the small-effect size in epidemiological analyses of the whole brain after corrections for multiple comparisons. Alternatively, very small Cu deficiencies disturb the maturation of the hippocampus and dentate gyrus (Hunt and Idso 1995), and Cu is prominently present in the basal ganglia areas and maybe particularly important in these areas. Similarly, for some areas, Cu deficiency may likely lead to disturbed development in specific regions.

In contrast, regarding the associations between high Cu levels, high MD values, and low cognitive functions, high Cu levels contribute to greater oxidative damage, which leads to neural damage, particularly in the substantia nigra (Segura-Aguilar et al. 2001) and increased accumulation of beta amyloid at later stages (Hashimoto 2008). Further, Cu

Table 3 Brain regions that exhibited significant positive correlations between the logarithms of Cu levels in the hair and rGMVs

Included gray matter areas[a] (number of significant voxels in the left and right sides of each anatomical area)	*x*	*y*	*z*	TFCE value	Corrected *p* value (FWE)	Cluster size (voxel)
Amygdala (L:462, R:529)/Angular gyrus (L:1042, R:3010)/Calcarine cortex (L:33, R:197)/Caudate (R:69)/Middle cingulum (L:1240, R:1699)/Posterior cingulum (L:582, R:363)/Cuneus (L:228, R:335)/Inferior frontal operculum (L:3)/Inferior frontal orbital area (L:746, R:1279)/Inferior frontal triangular (L:318)/Middle frontal orbital area (L:15, R:93)/Superior frontal orbital area (L:215, R:280)/ Superior frontal other areas (L:51, R:5)/Fusiform gyrus (L:6, R:85)/Heschl gyrus (L:216)/Hippocampus (L:1007, R:786)/Insula (L:2023, R:1500)/Lingual gyrus (R:1186)/Middle occipital lobe (L:2314, R:1321)/Superior occipital lobe (L:89, R:102)/Pallidum (L:94, R:1)/Paracentral lobule (L:1049, R:818)/Parahippocampal gyrus (L:482, R:798)/Inferior parietal lobule (L:1799, R:2117)/Superior parietal lobule (L:2302, R:1976)/Postcentral gyrus (L:1068, R:3026)/Precentral gyrus (L:639, R:285)/Precuneus (L:4903, R:3891)/Putamen (L:920, R:488)/Rectus gyrus (L:252, R:70)/Rolandic operculum (L:545, R:170)/Supplemental motor area (L:978, R:1289)/Supramarginal gyrus (L:1511, R:2721)/Inferior temporal gyrus (R:841)/Middle temporal gyrus (L:269, R:2091)/Temporal pole (L:1588, R:2197)/Superior temporal gyrus (L:1302, R:923)/Cerebellum (R:2)/	27	− 46.5	64.5	3177.48	< 0.001	79,611
None	−18	27	−7.5	1365.34	0.048	22

[a]Labelings of the anatomical regions of gray matter were based on the WFU PickAtlas Tool (http://www.fmri.wfubmc.edu/cms/software#PickAtlas/) (Maldjian et al. 2003, 2004) and on the PickAtlas automated anatomical labeling atlas option (Tzourio-Mazoyer et al. 2002). Temporal pole areas included all subregions in the areas of this atlas

Table 4 Statistical results (beta value, *t* value, uncorrected *p* values, *p* value corrected for FDR) for the multiple regression analyses performed using mean diffusivity (MD) or each region of interest and the copper level after correcting for confounding variables

Dependent variables	*N*	*β*	*t*	Copper level	
				p (uncorrected)	*p* (FDR)
MD of the right substantia nigra	924	0.082	2.624	0.009	0.035
MD of the left substantia nigra	924	− 0.010	− 0.303	0.762	0.332
MD of the right hippocampus	924	0.069	2.127	0.034	0.040
MD of the left hippocampus	924	0.068	2.131	0.033	0.040

The table presents beta values, *t* values, uncorrected *p* values, and *p* values corrected for FDR for multiple regression analyses performed using the mean diffusivity (MD) or each region of interest and Cu levels after correcting for confounding variables

FDR false discovery rate

triggers a proinflammatory state by modulating the production of molecules, such as IL-12, eventually injuring the tissue via this mechanism (Manto 2014).

Intranigral administration of Cu promotes the apoptosis of dopaminergic neurons (Manto 2014). MD of the brain may reflect the amount in various types of tissues in certain regions (Johansen-Berg et al. 2012); MD is sensitive to rapid neural plasticity (Sagi et al. 2012; Takeuchi et al. 2015a). Therefore, compared with rGMV, MD may be more sensitive to recent subtle changes in the brain. Perhaps the subtle damage to neural tissues, particularly in the hippocampus and resultant functional loss, may contribute to greater MD and cognitive functions. However, these are pure speculations. However, Cu levels were not associated with cognitive abilities, traits, and states associated with dopaminergic functions such as novelty seeking and extraversion (Takeuchi et al. 2013c). These cognitions were associated with MD of the basal ganglia, thalamus, and contingent regions, but not with MD of the substantia nigra (Takeuchi et al. 2015b, 2016b) and that may partly explain lack of the significant associations. Future studies involving animals may be required to reveal the mechanisms of the present associations.

Contrary to other cognitive functions, lower Cu levels were associated with seemingly worse cognitive function for reverse Stroop interference. However, because of the complex nature of reverse Stroop interference, it is unclear whether the results indicate that lower Cu levels are associated with aberrant neural function. In the present study, low Cu levels were associated with enhanced reverse Stroop interference, which indicates a diminished ability to resolve interference in the reverse Stroop task. However,

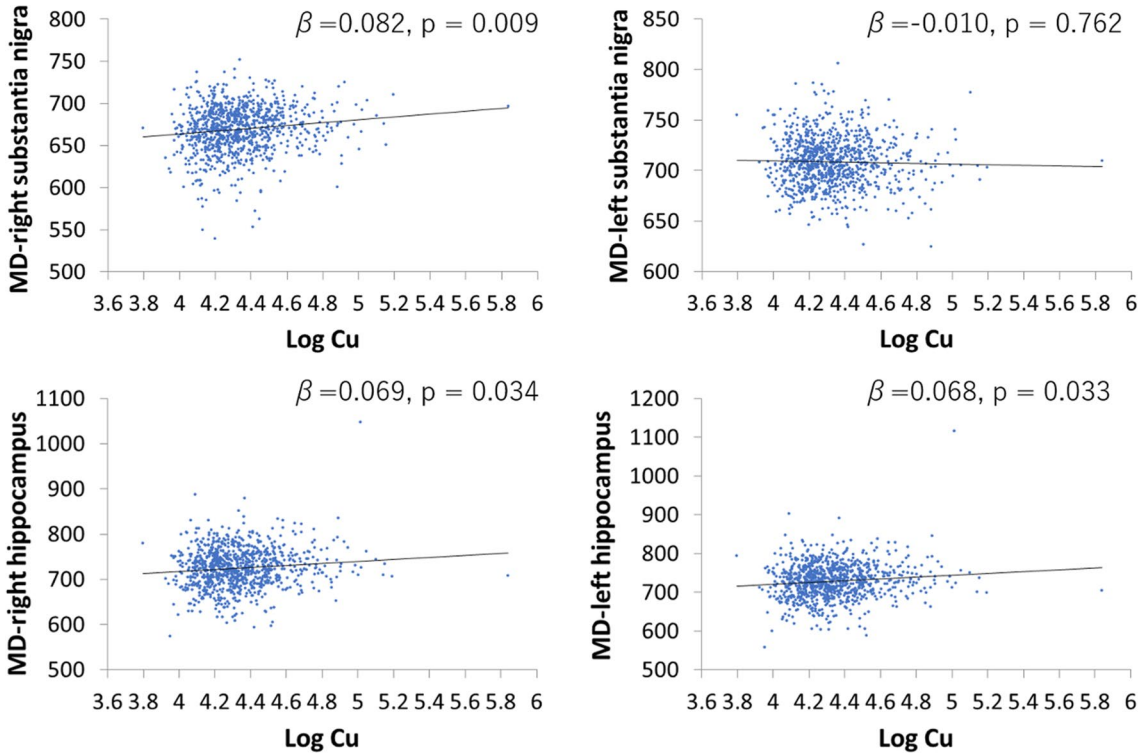

Fig. 3 Scatter plots of the associations between copper levels in the hair (logarithms of Cu levels in the hair) and the MD of the bilateral substantia nigra and bilateral hippocampus. Significant associations were found in the right substantia nigra and bilateral hippocampus, but not in the left substantia nigra. The statistical values are those of multiple regression analyses after correcting for confounding variables

the complex nature of this measure is known. First, the reverse Stroop effect is only observed for matching-type Stroop tasks (which we used in this study) and not for oral naming-type tasks. Further, the Stroop effect is observed for both tasks, and although patients with schizophrenia show enhanced Stroop interference and enhanced reverse Stroop interference, aging leads to enhanced Stroop interference and reduced reverse Stroop interference (Sasaki and Hakoda 1985; Sasaki et al. 1993).

Working memory training using mental calculations, which increases working memory capacity, leads to increased reverse Stroop interference (Takeuchi et al. 2011a). To our knowledge, it unclear why reverse Stroop interference shows this type of antinomic character (increase of interference in schizophrenic patients, increase of interference after cognitive training involving attention, but decrease in aging). One possibility may be related to the nature of the reverse Stroop task in which one must ignore the color of the letters but must see the next stimuli outside the center of the view to solve problems as fast as possible. The widened attention capacity (or field of view) may allow one to pay attention to the color characteristics of the stimuli outside the center of the vision, but one is unable to see the exact lettering of the word when the letter is outside the center of the view. Therefore, the facilitated neurocognitive function that widens the field of view may make it difficult to ignore the color of the letters. Future studies must show whether reduced Stroop interference in subjects with high Cu levels indicates aberrant neural function.

Although numerous studies have been devoted to the neurotoxicity of copper, we focused on such studies in the Introduction section. It is known that children with nutrition deficiency during prenatal development are more likely to develop obesity later in life due to epigenetic mechanisms (Tobi et al. 2014). Similarly, it is possible that copper deficiency during development may lead to neurocognitive maldevelopment and over-uptake of copper later in life and from the associations between two in the later in life. However, we believe this finding is hypothetical, and future studies need to test these ideas.

In conclusion, we investigated the associations of Cu levels in the hair with cognitive domains, rGMV, and MD in a relatively large cohort of young subjects. Studies on animals have demonstrated the important role of Cu in brain development as well as the toxicity of high Cu levels through multiple mechanisms. Human studies have shown the (a) associations of Cu levels in the hair with neurophysiology (Rimland and Larson 1983; Rahman et al. 2009; Priya and

Geetha 2011), (b) beneficial effects of Cu-chelating dietary components on neurophysiology (Squitti et al. 2002), and (c) the association of high cognitive decline in the elderly subjects associated with high Cu intake (Hashimoto 2008). Our novel findings showed the association of high Cu levels in the hair with (a) aberrant cognitive functions, (b) high rGMV in widespread regions of the brain, and (c) high MD in the hippocampus and substantia nigra even in healthy young adults, suggesting complex associations of Cu levels with neurocognitive mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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