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Localization of **copper and** copper transporters in the human brainKatherine M. Davies,^a Dominic J. Hare,^b Veronica Cottam,^a Nicholas Chen,^a Leon Hilgers,^a Glenda Halliday,^a Julian F.B. Mercer,^c and Kay L. Double^{*a}*Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX*

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Disturbances in brain copper result in rare and severe neurological disorders and may play a role in the pathogenesis and progression of multiple neurodegenerative diseases. Our current understanding of mammalian brain copper transport is based on model systems outside the central nervous system and no data are available regarding copper transport systems in the human brain. To address this deficit, we quantified regional copper concentrations and examined the distribution and cellular localization of the copper transport proteins Copper transporter 1, Atox1, ATP7A, and ATP7B in multiple regions of the human brain using inductively coupled plasma-mass spectrometry, western blot and immunohistochemistry. We identified significant relationships between copper transporter levels and brain copper concentrations, supporting a role for these proteins in copper transport in the human brain. Interestingly, the substantia nigra contained twice as much copper than that in other brain regions, suggesting an important role for copper in this brain region. Furthermore, ATP7A levels were significantly greater in the cerebellum, compared with other brain regions, supporting an important role for ATP7A in cerebellar neuronal health. This study provides novel data regarding copper regulation in the human brain, critical to understand the mechanisms by which brain copper levels can be altered, leading to neurological disease.

Introduction

Copper is an essential micronutrient and transition metal required for various cellular functions including cellular respiration, free-radical defense, neurotransmitter synthesis, neuronal myelination, and iron metabolism. In excess of cellular requirements, however, copper is toxic because it can participate in reactions that generate free radicals, thus contributing to oxidative stress.¹ Neuronal health and function thus depends on cellular mechanisms that regulate copper uptake, distribution, utilisation, storage, detoxification and efflux in the brain.

Compromised copper transport in the human brain is manifested as severe and well-characterized neurodegenerative disorders, including Menkes disease² and Wilson's disease.³ A growing body of evidence suggests that, in addition to these disorders, disturbances in the distribution of brain copper levels are associated with other neurodegenerative diseases,⁴ including Alzheimer's disease,^{5,6} amyotrophic lateral sclerosis (ALS),^{7,8} Parkinson's disease,^{9,10} Huntington's disease,^{10,19} and prion diseases.¹¹ Alterations in brain copper levels enhance cell death mechanisms in these disorders, including protein aggregation,¹²⁻¹⁴ oxidative stress^{5,15} and mitochondrial dysfunction,¹⁶ but the homeostatic control of copper in the human brain has not been determined.

To address this deficit, we describe here the associations between regional copper concentrations with the levels and cellular localization of major copper transport proteins Copper transporter 1 (Ctr1), Atox1, and the ATPases, ATP7A and ATP7B. An understanding of copper transport in the normal adult human brain will be essential for understanding diseases where brain copper levels are altered and for the development of

more effective treatments for these disorders.

Experimental

Brain tissues

Consent to use autopsy material for research purposes was obtained for all human brain samples. Tissues were received from New South Wales Tissue Resource Centre at the University of Sydney and the Sydney Brain Bank at Neuroscience Research Australia. Autopsy was performed within 48 hours of death and, to prevent metal contamination, brain tissues were dissected using polytetrafluoroethylene-coated blades (ProSciTech, Queensland, Australia). Fresh tissues were frozen immediately in liquid nitrogen, then stored at -80°C prior to analysis. Fixed tissues were stored in formalin. Ten human brains from subjects free of neurological disorders and neuropathological abnormalities were investigated. The details of these cases are given in Table 1. Brain regions investigated included the cerebellum, body of caudate, putamen, visual cortex, anterior cingulate cortex, and substantia nigra. Substantia nigra tissue was provided from only five of these ten human brains, as described in Table 1.

Materials

Primary antibodies used for detection of copper transport proteins by Western blotting, immunohistochemistry (IHC), and immunofluorescence (IF) are detailed in Table 2. Specificity of each antibody was confirmed by including no primary controls for Western blotting, IHC, and IF. ~~(Fig. 4).~~ Western blot assay was performed on brain tissue following preadsorption of the Atox1 antibody with the antigen against which it was raised, and

the ATPase antibodies have been well characterized in previous reports.^{17,18} Horseradish peroxidase-conjugated antibodies used for detection of proteins by Western blotting include: mouse anti-goat IgG, goat anti-mouse IgG, and donkey anti-sheep/goat IgG (all Millipore, USA). Secondary antibodies used for IHC include: Biotinylated horse anti-goat IgG (H+L) and biotinylated rabbit anti-sheep IgG (H+L) (both Vector Laboratories Inc., CA, USA). Vectastain ABC Kit Elite was used for IHC (Vector Laboratories Inc., CA). Donkey anti-goat Alexa Fluor® 594 IgG (H+L) and donkey anti-rabbit Alexa Fluor® 488 IgG (H+L), were purchased from Invitrogen (Eugene, Oregon, USA), for detection of Ctr1 and TH by IF, respectively.

Whole tissue metal content

Samples of frozen brain (34–70 mg) were subjected to closed-vessel microwave digestion (Milestone MLS1200) in 3 mL of concentrated nitric acid and 1 mL 35% hydrogen peroxide (both Seastar Chemicals, British Columbia, Canada). Digests were diluted w/w to c.a. 50 g with 1% nitric acid, and total metal concentrations were determined using an Agilent Technologies 7500cs inductively coupled plasma mass spectrometer (ICP-MS) (Forrest Hill, Victoria, Australia). Measured masses (*m/z*) included 55, 57, 63, 64, 65, 66 and 82, representing ⁵⁵Mn, ⁵⁷Fe, ⁶³Cu, ⁶⁴Zn, ⁶⁵Cu, ⁶⁶Zn, and ⁸²Se isotopes. Potential polyatomic interferences were removed using a He collision gas, specifically ⁴⁰Ar¹⁶OH⁺ on ⁵⁷Fe, ²³Na⁴⁰Ar⁺ on ⁶³Cu, and ³²S₂⁺ and ³²SO₂⁺ on ⁶⁴Zn. Routine tuning of the ICP-MS prior to use with a standard solution of ⁷Li, ⁵⁹Co, ⁸⁹Y, ¹⁴⁰Ce and ²⁰⁵Tl in a 1% HNO₃/HCl matrix. Matrix-based (*m/z* 94/59, ⁵⁹Co³⁵Cl⁺/⁵⁹Co⁺) and spectral (*m/z* 156/140, ¹⁴⁰Ce¹⁶O⁺/¹⁴⁰Ce⁺) interference formation was maintained at <1% at a He flow rate of 6 mL min⁻¹. Reported Cu and Zn concentrations are the average of *m/z* 63 and 65; and 64 and 66, respectively, with both metals conserving natural isotopic abundance ratios. Analytical performance was determined by measuring the detection limits (DL) and background equivalent concentration (BEC) for each measured mass (Table 1, ESI[†]). Mean elemental concentrations are shown in µg g⁻¹ wet weight ± standard error of the mean (SEM).

Preparation of brain tissue homogenate

Tissue homogenates were prepared in 15 volumes of buffer solution (10 mM Tris-HCl, 1% SDS, pH 7.5), protease inhibitor cocktail (Sigma) and leupeptin (25 µM final concentration) using a hand-held electronic homogenizer with a polycarbonate probe (OmniTH, Kelly Scientific, Australia). Homogenates were centrifuged at 15,700 g for 10 mins at 4 °C, supernatants collected and stored at -80 °C. Pierce® BCA Protein Assay Kit (Thermo Scientific) was used to determine sample protein concentration.

SDS-PAGE and Western Blot Analysis

The following protein amounts were loaded onto 4–12% Bis-Tris Criterion pre-cast gels (Bio-Rad); Ctr1: 40 µg, Atox1: 80 µg, ATP7A: 120 µg, ATP7B: 120 µg, and separated by SDS-PAGE in MES (Atox1) or MOPS (Ctr1, ATP7A, ATP7B) buffer (Bio-Rad), according to manufacturer's instructions (Bio-Rad). Separated proteins were transferred to Immobilon- P^{5Q} PVDF

(Millipore) (Ctr1, Atox1, ATP7A) or nitrocellulose (Millipore) (ATP7B) membranes. Following transfer, membranes were blocked in either 5% skim milk (Ctr1, ATP7A, and ATP7B), or 1% casein (Atox1), in PBS containing 0.1% Tween® 20 and incubated with appropriate primary antibodies diluted in PBS containing 0.1% Tween® 20 and 1% skim milk (Ctr1, ATP7A, and ATP7B) or 1% casein (Atox1) at the following concentrations: Ctr1 (1:200), Atox1 (1:250), ATP7A (1:750), and ATP7B (1:1500) overnight at 4 °C. Following incubation with horseradish peroxidase-conjugated mouse anti-goat IgG (1:10,000), goat anti-mouse IgG (1:2,000), or donkey anti-sheep/goat IgG (1:2,000), protein signals were obtained using an ECL Western blotting detection system (Bio-Rad) as per the manufacturer's instruction's, and developed using the Chemi-Doc XRS (Bio-Rad). Signal intensities were quantified by densitometry using Quantity One® software (Bio-Rad) and normalized to β-actin (1:10,000) levels.

Immunohistochemistry

Formalin-fixed slide-mounted paraffin-embedded sections (7 µm) were antigen retrieved by the following methods: Tris-EDTA buffer (0.01M Tris, 1 mM EDTA, pH 9.0) at 95 °C for 20 mins (Ctr1), citrate buffer (pH 6.0) microwave irradiation for 15 mins (ATP7A and ATP7B). Non-specific peroxidase was blocked with 1% H₂O₂ (Fronine Laboratory Supplies) in 50% ethanol for 30 mins at room temperature. Non-specific antigen sites were blocked with a mixture of 1% bovine serum albumin (BSA; Sigma) and 5% normal horse serum (NHS; Australis) (Ctr1), Animal Free Blocker™ (Vector) (ATP7A), or 3% BSA (Sigma) (ATP7B), for 20 mins at room temp. Primary antibodies for IHC were used at the following dilutions: Ctr1 (1:100), ATP7A (1:300), ATP7B (1:1,000). Primary antibodies for Ctr1, ATP7A and ATP7B were detected using biotinylated IgG antibodies (Vector) (1:200), followed by Vector Elite Kit tertiary antibody complex (Vector) (1:100), and visualized using 3,3'-diaminobenzidine (DAB; Sigma) with a cresyl violet counterstain (Sigma-Aldrich Co.). As prominent expression of Ctr1 and ATP7A proteins have previously been reported in mouse choroid plexus using IHC,^{19,20} the choroid plexus of a single additional case was used as a positive control for all IHC experiments. Images were taken using a Zeiss AxioCam HRC microscope.

Immunofluorescence

Due to the inherent difficulty to separate positive DAB staining from neuromelanin, IF staining was performed on midbrain tissue sections to examine the cellular localization of Ctr1, ATP7A, and ATP7B in substantia nigra neurons. Formalin-fixed free-floating midbrain sections (50 µm; Ctr1) and formalin-fixed slide-mounted paraffin-embedded sections (7 µm; ATP7A and ATP7B) were antigen retrieved in Tris-EDTA buffer (0.01 M Tris, 1 mM EDTA, pH 9.0) at 95 °C for 25 mins (Ctr1), or citrate buffer (pH 6.0) with microwave irradiation for 15 mins (ATP7A and ATP7B), and permeabilized with 50% ethanol. Non-specific antigen sites were blocked in 10% NHS (Australis; Ctr1), or 0.25% Casein (Sigma; ATP7A and ATP7B) for 1 hour at room temperature and midbrain sections double-stained for

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the dopaminergic cell marker tyrosine hydroxylase (TH; 1:1000) and for Ctrl (1:25), ATP7A (1:100), or ATP7B (1:400). Primary antibodies were detected using secondary IgG antibodies conjugated to Alexa Fluor® 594 or Alexa Fluor® 488 fluorophores (Invitrogen), respectively and images were taken under high magnification, using a Nikon D-Eclipse C1 Si scanning confocal microscope.

Statistical Analyses

Statistical analysis was performed using PASW v18 (SPSS inc.). Univariate analysis of variance followed by Bonferroni's t-test was used to test for relationships between copper transporter or copper levels and tissue variables including brain region, brain pH, post-mortem delay, and **gendersex**. Linear regression was used to test for relationships between copper transporter levels, copper levels, and tissue variables with age. The significance level was set at 0.05.

Results and discussion

Regional metal levels in the human brain

In addition to total copper levels, regional metal levels determined by ICP-MS are shown in Table 3. **Analytical figures of merit (see Table 1, ESI demonstrate adequate sensitivity for determining metal concentrations in tissue digests.** With the exception of selenium, metal levels varied in a regional manner unique for each metal and were unaffected by post-mortem delay, brain pH, or **gendersex**. Copper levels in all brain regions analyzed were remarkably similar, varying between 4 and 5 µg of copper/g of wet weight tissue, with the exception of the substantia nigra, which contained copper levels more than two-fold higher than that in all other brain regions analysed ($p < 0.001$ for every brain region; Table 3). ^{9,21,22,23} Zinc levels were slightly higher than copper levels, as previously reported,²¹ and were significantly greater in the substantia nigra, compared to the putamen ($p = 0.04$), visual cortex ($p = 0.026$) and anterior cingulate cortex ($p = 0.038$) (Table 3). Consistent with previous reports,^{9,21} iron was the most abundant metal in all regions and levels were greatest in the substantia nigra, followed by the body of caudate and putamen (Table 3). Iron levels were significantly higher in the substantia nigra and body of caudate compared with the visual cortex ($p = 0.006$, $p = 0.029$), anterior cingulate cortex ($p = 0.001$, $p = 0.006$), and the cerebellum ($p < 0.001$, $p = 0.002$), respectively. **These data suggests an increased requirement for, or storage of copper, zinc, and iron in the substantia nigra. This brain region, associated with high levels of oxidative stress, may require high levels of copper and zinc for the function of Cu/Zn superoxide dismutase, a copper- and zinc-dependent enzyme critical for antioxidant defense.** **Iron is required for TH activity, the rate-limiting step in dopamine synthesis.**²³ **Further, some studies suggest that these metals are also required for**

neuromelanin synthesis.²⁴⁻²⁷ Manganese and selenium levels were markedly lower than copper, zinc, and iron levels and, while manganese levels were greatest in the body of caudate and lowest in the anterior cingulate cortex ($p = 0.028$), selenium levels did not differ between brain regions (Table 3). Copper, iron and zinc levels did not significantly change with age, but levels of manganese and selenium significantly increased with age ($p = 0.043$, and $p = 0.003$, respectively). Selenium accumulated uniformly in all brain regions, whereas the age-related increase in manganese primarily resulted from an increase in the body of caudate ($p = 0.031$).

Copper transporter protein levels

Western blotting detected Ctrl (28kDa), Atox1 (7kDa), ATP7A (173kDa), and ATP7B (165kDa) in all brain regions (Fig. 1). Regional levels of Ctrl, Atox1 and ATP7B did not differ, but levels of ATP7A in the cerebellum were significantly greater than that in all other brain regions investigated ($p = 0.004$), with the exception of the substantia nigra (Table 4). Regression analyses identified significant relationships between both Ctrl and Atox1 protein levels and brain copper levels ($p = 0.035$ and $p = 0.002$, respectively; Fig. 2). There was no relationship between ATP7A, or ATP7B levels and brain copper levels, nor were levels of any of the copper transporter proteins associated with each other or with age.

Copper transporter cellular localization

IHC of copper transporter proteins demonstrated expression of Ctrl, ATP7A, and ATP7B in all brain regions in distinct cell populations (Fig. 3). Although we detected quantifiable amounts of Atox1 by western blot, **consistent with studies in human cell lines and liver,**²⁸ this protein was not able to be visualized using IHC **on fixed paraffin-embedded tissue. There is only one report, where Atox1 protein has been detected by IHC, using an in-house Atox1 antibody.**²⁹ **Atox1 is a small soluble cytoplasmic 7kDa protein and is not membrane bound. Therefore, it is likely that this soluble protein is lost during tissue fixation and antigen retrieval processes where soluble solutions are exchanged between internal and external tissue compartments. Many small water-soluble transmitters, for example, are not visible using IHC in fixed tissues (some refs here?).**

Intense cytoplasmic staining for Ctrl protein, particularly concentrated to the apical surface, was observed in the polarized epithelial cells of the choroid plexus (Fig. 3A). **Apical and basolateral membranes of the choroid plexus were determined based on morphology, as described in previous studies.**³⁰⁻³³ Ctrl staining in the visual cortex, anterior cingulate cortex, and caudate and putamen (Fig. 3D, G and M), however, revealed a punctate distribution in the cytoplasm, which differed to that

Comment [GH1]: Was this tissue fixed, or cut frozen and fixed on slide?

Comment [KS2]: Glenda, do you think this is a fair suggestion?

seen in the choroid plexus. While Ctr1 staining in the visual cortex, anterior cingulate cortex, and caudate and putamen was primarily neuronal (Fig. 3D, G and M), in the cerebellum there was a striking lack of expression of Ctr1 in Purkinje neurons (Fig. 3J). Rather, Ctr1 expression was restricted to Bergmann glia (Fig. 3J). In the anterior cingulate cortex, Ctr1 staining of the proximal fibers was very weak compared to staining in neuronal cell bodies (Fig. 3G). Ctr1 staining of the striosomes in the caudate and putamen was also very weak (Fig. 3M).

—Substantia nigra pars compacta (SNpc) dopaminergic neurons exhibited robust Ctr1 staining in a circular pattern around the neuromelanin granules (Fig. 4A). The non-pigmented substantia nigra pars reticulata (SNpr), was predominantly negative for Ctr1.

Cytoplasmic and apical membrane staining for ATP7A was observed in the polarized epithelial cells of the choroid plexus, ~~and but~~ was most concentrated ~~at~~ the basolateral surface (Fig. 3B). In contrast, ATP7B protein was expressed predominantly in the membrane of these epithelial cells and was most concentrated on the apical surface (Fig. 3C). In all other brain regions investigated, the staining pattern for ATP7A was very similar to ATP7B. ATP7A and ATP7B staining in the visual cortex and anterior cingulate cortex (Fig. 3E, F, H and I) was localized to the cytoplasm. Unlike Ctr1 staining in these brain regions, ATP7A and ATP7B staining was not punctate. Most notably, while Ctr1 was strongly expressed only in neuronal cell bodies in the anterior cingulate cortex, ATP7A and ATP7B were strongly expressed in both neuronal cell bodies and proximal fibers in this brain region (Fig. 3H and I), and, unlike Ctr1, ATP7A and ATP7B, staining was clearly positive in the striosomes of the caudate and putamen (Fig. 3N and O). Furthermore, strong staining for the ATPases was observed in cerebellar Purkinje neurons, and not in Bergmann glia (Fig. 3K and L).

—In contrast to Ctr1, staining for ATP7A was not specifically localized to neuromelanin in the SNpc, but was most strongly expressed in the cytoplasm (Fig. 4B). ATP7B was present in the cytoplasm and associated with neuromelanin (Fig. 4C). Both ATP7A and ATP7B were not present in the nucleus or axons (Fig. 4B and C).

Ctr1,¹⁹ ATP7A,^{20,34,35} and ATP7B^{34,35} proteins ~~and mRNA~~³⁶ have been previously reported in rodent brain, while Atox1 protein has been identified only in human cell lines and human liver.²⁸ Here we report the presence of the copper transporter proteins Ctr1, Atox1, ATP7A, and ATP7B in the human brain.

Few studies have analysed regional variations in copper levels in the normal human brain.^{9,21,37,38} Consistent with previous reports,^{9,37,39} copper levels were highest in the human substantia nigra, suggesting an increased requirement for, or storage of, copper in this brain region.

We demonstrate here that regional levels of the major copper transport protein Ctr1 are associated with regional differences in copper levels. While descriptive in nature, these data are consistent with data from experimental animal models reporting elevated brain Ctr1 protein levels in Cu deficient mice,^{19,40} and support a role for this protein in regulating copper levels in the human brain. Western blot analyses demonstrated that the

primary form of Ctr1 in the human brain is the monomeric (28kDa) protein,^{41,42} previously reported in mouse choroid plexus.⁴⁰

Ctr1 was observed to have a cytoplasmic, vesicular distribution, in agreement with findings in human placental tissue⁴³ and a number of human carcinoma cell lines.⁴² It has been hypothesized that Ctr1 can exist as both intracellular and plasma membrane pools of protein in a state of pseudo-equilibrium, which change in response to variations in extracellular copper levels.^{44,45} Our data suggest that, in the normal human brain with adequate cellular copper, Ctr1 exists primarily as an internalized protein pool, rather than as an active membrane-bound transporter. This is consistent with *in vitro* data from HEK cells over-expressing human Ctr1 which showed that the protein is internalized from the plasma membrane in response to extracellular copper levels within or above the normal physiological range, but recycled to the plasma membrane when extracellular copper levels are depleted.⁴⁴

While Ctr1 was localized to the cytoplasm in non-pigmented midbrain neurons, in pigmented neurons Ctr1 was associated with neuromelanin granules (Fig. 4A) and may regulate the acquisition of copper by this pigment.⁴⁶ In support of this hypothesis, *Drosophila* expressing a mutant form of the high affinity copper transporter protein, Ctr1B, exhibit decreased larval copper accumulation and body pigmentation defects.⁴⁷

Like Ctr1, levels of Atox1 protein were associated with regional copper levels, suggesting this protein also plays a role in regulating brain copper. These findings are in agreement with a previous report of Atox1 mRNA in human brain⁴⁸ and the expression pattern of Atox1 mRNA in the rat brain, where the highest levels of Atox1 mRNA are found in neuronal populations that sequester copper.⁴⁹

With the exception of the polarized epithelial cells of the choroid plexus, ATP7A and ATP7B were predominantly localized to the cytoplasm, consistent with their proposed localization to the trans-Golgi network.⁵⁰⁻⁵² In addition to neuronal cytoplasmic staining, strong expression of ATP7A and ATP7B proteins, but not of Ctr1, was observed in the cerebellum, anterior cingulate cortex, and caudate putamen in the proximal fibers. A role for ATP7A in proximal fiber health is supported by studies in mouse models of Menkes' disease, a lethal infantile neurodegenerative disorder of copper metabolism caused by mutations in ATP7A. In these animals, axons in the cerebral cortex, cerebellum, and hippocampus remain undeveloped, and cortical dendrites are swollen,^{35,53} but this pathology is avoided by ATP7A gene therapy at the choroid plexus, plus copper treatment.⁵³ Interestingly, ATP7A and ATP7B were not expressed in the axons of dopaminergic neurons in the substantia nigra, suggesting that in the normal adult brain these axons do not actively require ATP7A expression. This concept of a stage-specific role for axonal ATP7A is supported by studies in the mouse brain where ATP7A protein expression in the dendrites of Purkinje neurons was demonstrated to increase into adulthood, but was lost in the optic nerve axons of postnatal mice.²⁰

Interestingly, neither ATP7A nor ATP7B protein levels were significantly associated with brain copper levels. This finding is in agreement with previous studies demonstrating that changes

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in copper concentration have no effect on steady state protein levels of ATP7A *in vitro*⁵⁰ or ATP7B *in vivo*,⁵¹ but that copper levels influenced the subcellular localization of these proteins.^{50,51} These data suggest that, in the adult human brain, changes in copper levels may be regulated by changes in the cellular localization of the ATPase proteins, rather than changes in their level of expression.

While Ctr1, ATP7A and ATP7B proteins were observed to be localized to the cytoplasm in most brain regions, the polarized epithelial cells of the choroid plexus exhibited unique patterns of staining, suggesting that copper is transported in the choroid plexus in a way that is distinct from other brain regions. ATP7A was localized to the cytoplasm and the cell membrane, where unlike ATP7B, it was noticeably present at the basolateral surface, consistent with its localization in polarized murine intestinal epithelial cells⁵⁴ and kidney.⁵⁵ ATP7B was predominantly localized to the cell membrane and was particularly concentrated at the apical surface, consistent with its localization in human breast epithelial cells⁵⁶ and in agreement with *in vitro* data from polarized hepatocytes.⁵⁷ Although Ctr1 was predominantly cytoplasmic, it too was more concentrated at the apical surface. The observed localization for ATP7A and ATP7B suggests a role for ATP7A in mediating copper efflux across the basolateral membrane into capillaries, and suggests that ATP7B may mediate the transfer of copper into the CSF. This transfer may occur via a mechanism similar to that seen in mammary epithelial cells, where ATP7A is proposed to play a role in removing excess copper, while ATP7B transports copper across the apical surface and is thought to be the major means of copper secretion into milk during lactation.⁵⁸

In selected brain regions the ATPases and Ctr1 were expressed in different cell types. In the cerebellum, for example, Ctr1 was strongly expressed in Bergmann glia but not detected in Purkinje neurons, whereas the ATPases demonstrated an inverse pattern of expression. Cell-type specific expression has previously been demonstrated in rat dorsal root ganglion⁵⁹ and human placental tissue,⁴³ where cells that strongly expressed ATP7A, expressed lower levels of Ctr1. Increased Ctr1 expression in mammary tissue during pregnancy and lactation,¹⁹ and in duodenal and brain tissue from copper deficient mice,¹⁹ suggest that Ctr1 expression may depend on the immediate copper requirement of the cell. Further, changes in the intensity of Ctr1 staining and the cell-type specific expression of the ATPases have been demonstrated in mouse brain tissue at different stages of development.^{19,34} It has been suggested that cells which strongly express Ctr1 have a high requirement for copper potentially to meet cuproenzyme requirements,^{60,62} while cells with low Ctr1 expression and high ATP7A may be sensitive to copper and require a high copper efflux capacity.⁵⁹ ATP7A expression was greatest in the cerebellum where staining was localised to the Purkinje neurons, a finding also reported in the mouse brain.²⁰ Higher levels of ATP7A may be required in

the cerebellum to maintain normal function, as the cerebellar Purkinje neurons are particularly sensitive to copper changes in Menkes' disease.⁶³⁻⁶⁵

Conclusions

In this first comprehensive study of copper transporter proteins in the human brain, we have described the presence of Ctr1, Atox1, ATP7A, and ATP7B in multiple human brain regions and identified significant relationships between brain copper and copper transporter protein levels. An understanding of cellular copper transport in the human brain will be critical to understand the mechanisms of disrupted brain copper in multiple disorders where brain copper levels are altered.

Disclosure

The authors report no conflicts of interest.

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⁹⁵ † Electronic Supplementary Information (ESI) available. See DOI: 10.1039/b000000x/

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Figure legends

Fig. 1 Representative Western blots demonstrating the presence of the major copper transport proteins Copper transporter 1 (Ctr1), Atox1, ATP7A, and ATP7B in multiple regions in the human brain, including the substantia nigra (SN), anterior cingulate cortex (ACC), visual cortex (VC), putamen (P), body of caudate (C), and cerebellum (CB). β -actin was used as a protein loading control.

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Fig. 2 Regression analyses demonstrated a significant association between both normalized Copper transporter 1 (Ctr1) levels and Atox1 levels of copper transport proteins Ctr1 and Atox1 were significantly associated with copper (Cu) levels in the human brain. Copper transporter levels were quantified by densitometry performed on Western blots and normalized to β -actin levels (used as a loading control). Cu levels were determined by inductively coupled plasma-mass spectrometry.

Fig. 3 The cellular localization of copper transport proteins Copper transporter 1 (Ctr1), ATP7A, and ATP7B in the human brain. 7 μ m paraffin embedded human brain sections were probed for copper transporters, Ctr1, ATP7A, and ATP7B, and visualised using DAB peroxidase immunohistochemistry (brown) with cresyl violet counterstain. Regions of positive staining are dark brown. Scale bar in L applies to A-L. Scale bar in O applies to M-O. Insets show localization of copper transport proteins in the choroid plexus and caudate putamen at higher magnification. Arrows in A–C insets highlight more concentrated positive staining on the apical (A and C) and basolateral (B) membranes.

Fig. 4 Representative high magnification photomicrographs of the cellular location of Copper transporter 1 (Ctr1), ATP7A, and ATP7B in the human substantia nigra pars compacta. Midbrain sections dual stained for the dopaminergic marker, tyrosine hydroxylase (green), and Ctr1 (A), ATP7A (B), or ATP7B (C) (red). Yellow regions represent co-localisation of TH (green) with Ctr1, ATP7A, or ATP7B (red). Insets: No Ctr1 primary control (A), and no ATP7A and ATP7B primary control (C). Arrows highlight regions of positive staining.

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Tables

Case	Age (years)	Gender Sex	Post-mortem interval (hours)	Brain pH	Cause of death
1 [*]	41	Male	48	6.96	Ischaemic heart disease
2 [*]	49	Male	47	6.5	Coronary atherosclerosis
3 [*]	65	Male	14.5	6.79	Ischaemic heart disease
4	79	Male	8	6.65	Pulmonary embolism
5	84	Female	6	6.51	Respiratory arrest
6	85	Female	23	6.44	Pneumonia
7 [*]	92	Female	5	6.08	Pancytopenia
8	93	Female	21	6.96	Cardiac failure
9	97	Female	16	Not available	Pneumonia
10 [*]	102	Female	5	5.92	Acute renal failure

Table 1. Case details. All cases had no significant neuropathology at autopsy. Substantia nigra tissue was provided from cases marked with an asterisk^{*}.

<i>Antigen</i>	<i>Immunogen</i>	<i>Source</i>
Ctr1	Raised against a peptide mapping within amino acids 100-150 of CTR1 of human origin (accession number O15431).	Santa Cruz Biotechnology, Inc. (CA, USA); CTR1 (G-15); Cat. # sc-18473; goat polyclonal.
Atox1	ATOX1 (NP_004036, 1 a.a. – 68a.a.) partial recombinant protein with GST tag.	Abnova (Taiwan); ATOX1, clone 2E6; Cat. # H00000475-M01; mouse monoclonal.
ATP7A	Raised against the first 590 N-terminal amino acids of the human MNK protein.	Provided by Julian Mercer, Deakin University, Melbourne, Australia; R17; sheep polyclonal ¹⁸ .
ATP7B	Raised against a 36-kDa fusion protein with a His6 tag at the N-terminus of the protein comprising amino acids 1-199 (N-terminal), 1309-1315 and 1376-1465 (C-terminal) of the human ATP7B protein.	Provided by Julian Mercer, Deakin University, Melbourne, Australia; NC36; sheep polyclonal ¹⁷ .
β -actin	Raised against amino acids 50-70 of purified chicken gizzard actin.	Millipore (Billerica, MA, USA); Anti-Actin, clone C4; Cat. # MAB1501; mouse monoclonal
TH	Raised against amino acids 1-196 of TH of human origin.	Santa Cruz Biotechnology, Inc. (CA, USA); TH (H-196); sc-14007; rabbit polyclonal.

Table 2. Primary antibodies used in this study.

		<i>Metal ion levels (µg/g wet tissue weight)</i>				
		<i>Copper</i>	<i>Iron</i>	<i>Zinc</i>	<i>Manganese</i>	<i>Selenium</i>
<i>Brain region</i>	<i>n</i>					
Visual cortex	10	4.14±0.34	51.61±3.19	11.01±0.88	0.15±0.04	0.14±0.03
Anterior cingulate cortex	10	4.04±0.27	42.51±2.61	11.24±0.67	0.09±0.04	0.09±0.03
Body of caudate	10	5.09±0.70	108.01±20.60*	12.44±0.94	0.31±0.06*	0.07±0.03
Putamen	10	4.47±0.47	78.15±16.85	11.26±0.50	0.22±0.05	0.12±0.05
Substantia nigra	5	11.40±2.50*	131.97±15.57*	16.81±3.56*	0.20±0.05	0.07±0.04
Cerebellum	9	4.85±0.57	33.23±3.71	11.84±0.58	0.19±0.05	0.18±0.05

Table 3. Regional differences in metal levels, determined by ICP-MS. Values are mean ± SEM. Brain regions where metal levels were significantly greater than other regions are marked with an asterisk*. Levels of copper in the substantia nigra were greater than that in all other brain regions (p<0.001). Iron levels were higher in the substantia nigra and body of caudate compared with the visual cortex (p=0.006, p=0.029), anterior cingulate cortex (p=0.001, p=0.006), and cerebellum (p<0.001, p=0.002), respectively. Levels of zinc in the substantia nigra were greater than that in the putamen (p=0.04), visual cortex (p=0.026), and anterior cingulate cortex (p=0.038). Levels of manganese in the body of caudate were greater than that in the anterior cingulate cortex (p=0.028).

		Copper transporter levels (Relative to control)			
		<i>Ctrl</i>	<i>Atox1</i>	<i>ATP7A</i>	<i>ATP7B</i>
<i>Brain region</i>	<i>n</i>				
Visual cortex	10	0.65±0.07	1.08±0.21	0.67±0.12	0.58±0.19
Anterior cingulate cortex	10	0.68±0.09	1.34±0.25	0.88±0.18	0.47±0.19
Body of caudate	10	0.74±0.08	1.26±0.20	0.72±0.15	0.84±0.32
Putamen	10	0.71±0.12	1.39±0.22	0.70±0.28	0.61±0.20
Substantia nigra	5	0.73±0.16	2.05±0.60	1.00±0.22	0.33±0.15
Cerebellum	10	0.68±0.07	0.92±0.12	2.00±0.45*	0.78±0.27

Table 4. Regional differences in copper transporter levels, determined by Western blot. Copper transporter protein levels were semi-quantified by densitometry analysis of Western blot bands. Copper transporter levels were normalized to Beta-actin levels and expressed relative to an internal standard control. Values are mean ± SEM. Levels of ATP7A in the cerebellum were greater than that in all other brain regions (p<0.001)*.

Figures

Fig. 1

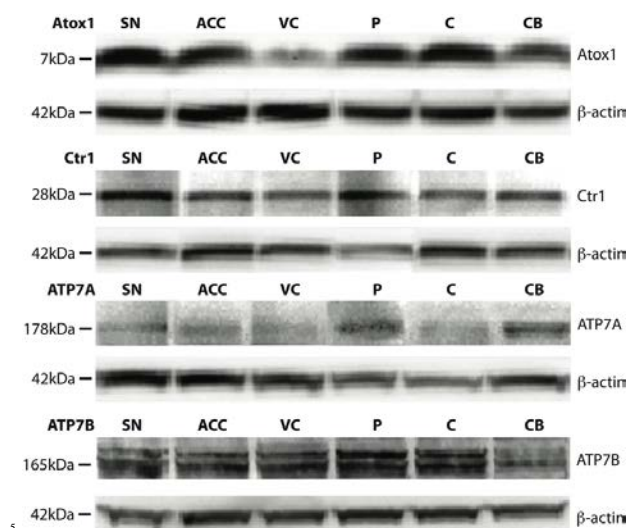
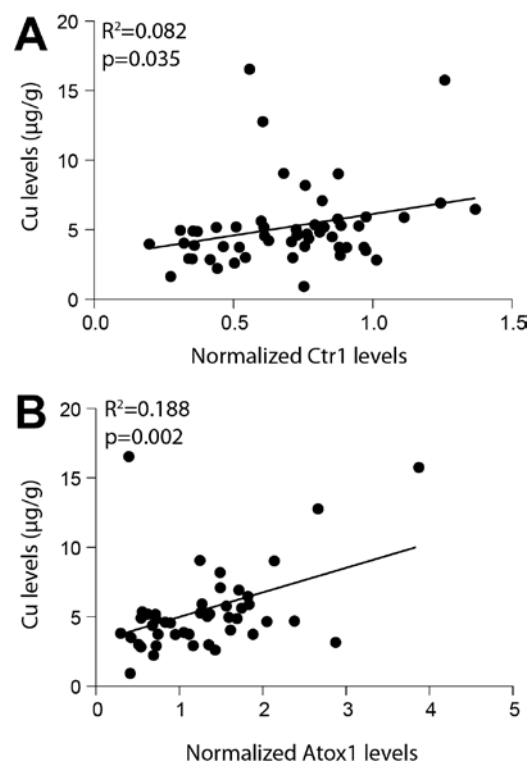


Fig. 2



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Fig. 3

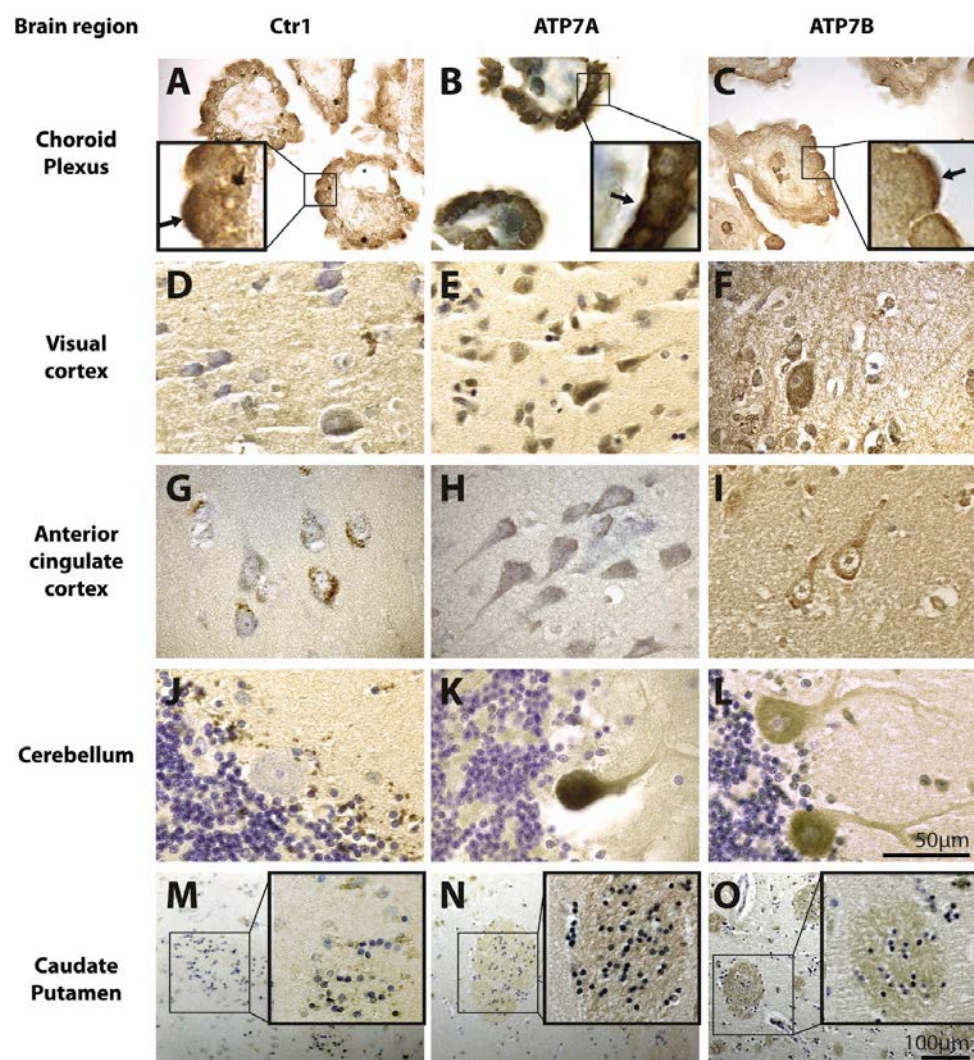
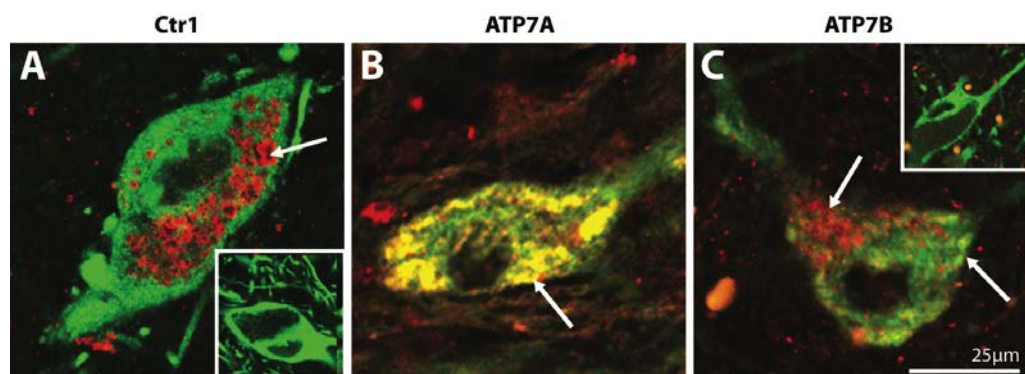


Fig. 4



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Supplementary Material

Mass	DL ($\mu\text{g kg}^{-1}$)	BEC ($\mu\text{g kg}^{-1}$)
^{55}Mn	0.008	3.110
^{57}Fe	0.478	2.879
^{63}Cu	0.278	4.559
^{64}Zn	0.288	3.987
^{65}Cu	0.303	4.607
^{66}Zn	0.561	6.867
^{82}Se	0.378	2.256

Supplementary Table 1 Detection limits (DL) and background equivalent concentration (BEC) for each mass measured by ICP-MS.