Presenilins Promote the Cellular Uptake of Copper and Zinc and Maintain Copper/Chaperone of SOD1-dependent Superoxide Dismutase Activity

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Dyshomeostasis of extracellular zinc and copper has been implicated in β-amyloid aggregation, the major pathology associated with Alzheimer disease. Presenilins mediate the proteolytic cleavage of the β-amyloid precursor protein to release β-amyloid, and mutations in presenilin can cause familial Alzheimer disease. We tested whether presenilin expression affects copper and zinc transport. Studying murine embryonic fibroblasts (MEFs) from presenilin knock-out mice or RNA interference of presenilin expression in HEK293T cells, we observed a marked decrease in saturable uptake of radiolabeled copper and zinc. Measurement of basal metal levels in 6-month-old presenilin 1 heterozygous knock-out (PS1+/−) mice revealed significant deficiencies of copper and zinc in several tissues, including brain. Copper/zinc superoxide dismutase (SOD1) activity was significantly decreased in both presenilin knock-out MEFs and brain tissue of presenilin 1 heterozygous knock-out mice. In the MEFs and PS1+/− brains, copper chaperone of SOD1 (CCS) levels were decreased. Zinc-dependent alkaline phosphatase activity was not decreased in the PS null MEFs. These data indicate that presenilins are important for cellular copper and zinc turnover, influencing SOD1 activity, and having the potential to indirectly impact β-amyloid aggregation through metal ion clearance.

Presenilins are novel membrane aspartyl proteases, characterized for their role as the catalytic subunit of γ-secretase, a multiprotein complex that mediates intramembrane proteolysis of numerous type-1 integral membrane proteins including the amyloid precursor protein (APP)3 and Notch-1 (1, 2). Two closely related genes, PSEN1 and PSEN2, were identified through the association of missense mutations with early-onset familial Alzheimer disease. These mutations alter processing of the amyloid precursor protein that generates the C terminus of the β-amyloid (Aβ) peptide, which contributes to the pathogenesis and hallmark plaque pathology of Alzheimer disease (AD). In terms of biological function, the ubiquitous presenilins have been implicated in a wide variety of cellular processes such as protein trafficking, signal transduction and signaling, cell adhesion, calcium homeostasis, and Tau phosphorylation (3).

Copper is essential for the activity of several enzymes including SOD1, a major antioxidant. Excess copper, however, can catalyze reactive oxygen species generation. Therefore, homeostatic machinery maintains essential, yet nontoxic, intracellular copper concentrations. Import of copper into mammalian cells is predominantly handled by copper transporter 1, a ubiquitously expressed transmembrane protein (4). However, ~30% of cellular copper import occurs by uncharacterized mechanisms (5).

Zinc, another vital biometal, is essential for more than 300 enzymes (including SOD1) and serves structural and antioxidative roles (6). Like copper, zinc ions cannot cross biological membranes by passive diffusion, and most zinc transport in mammals is carried out by two families of zinc transporters: ZIP (SLC39) and ZnT (SLC30) (7). ZnT3, a key zinc transporter in relation to AD, loads synaptic vesicles of glutamatergic neurons with Zn2+, which is then released during neurotransmission (8, 9).

Both Cu2+ and Zn2+ are released as freely exchangeable chemical species into glutamatergic synapses where they can reach concentrations of 300 and 15 μM, respectively (10), and may function as neuromodulators (11, 12). Therefore Aβ, zinc, and copper co-localize in the synaptic vicinity where they appear to interact in AD to form oxidized, cross-linked soluble aggregates and precipitated amyloid (10). Consistent with this mechanism, amyloid pathology in APP transgenic mice (13) and the adherence of soluble Aβ oligomers to NR2B NMDA receptor subunits (14) are both markedly inhibited in the absence of ZnT3. Because these interactions are important in AD pathogenesis, we investigated whether the expression of PS itself may impact on copper and zinc homeostasis. In the current study we identify a novel role for PS in cellular copper and zinc uptake and demonstrate its impact on the copper- and zinc-dependent activity of SOD1.

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3 The abbreviations used are: APP, amyloid precursor protein; Aβ, amyloid β; Aph1, anterior pharynx-defective 1; CCS, copper chaperone of SOD1; DAPT, N-(3,5-difluorophenacetyl-L-alanyl)-S-phenylglycine t-butyl ester; dKO, double knock-out; HBSS, Hank’s balanced salt solution; MEF, mouse embryonic fibroblast; PS, presenilin; SOD1, superoxide dismutase 1; AD, Alzheimer disease; AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; ALP, alkaline phosphatase; h, human.

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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Mice**—Mouse embryonic fibroblast (MEF) cells were generously provided by Dr. Bart De Strooper and have been described (15). Low passage MEF and human embryonic kidney (HEK293T) cells were cultured in Dulbecco’s modified Eagle’s medium (SAFC Biosciences, Lenexa, KS), supplemented with 10% fetal bovine serum (Bovogen, Keilor East, Australia) and 0.058% L-glutamine. The cells were maintained at 37 °C with 5% CO₂. Heterozygote PSEN1 knock-out mice was on C57BL/6 background strain and have been described previously (16). Wild type, age-matched (6 months old) littermates were used as controls. The mice were housed in a registered facility, and all of the animal procedures were approved by the University of Melbourne animal ethics committee (Register number 06169).

**Radiolabeled Metal Uptake Assays**—⁶⁴Cu uptake experiments were adapted from Camakaris et al. (17). The uptake media contained Hank’s balanced salt solution (HBSS), supplemented with 0.4MBq of⁶⁴CuCl₂ (Australian Radiopharmaceuticals and Industrials) and unlabeled CuCl₂. Copper uptake was stopped by washing cell monolayers three times with ice-cold nonlabeled HBSS (with 2 mM L-histidine). The cells were then harvested with a rubber cell scraper in 0.1% SDS with 2 mM EDTA and transferred to microcentrifuge tubes. ⁶⁴Cu was measured using a γ-counter (1282 CompuGamma, LKB Wallac). Copper levels were standardized to total cellular protein, measured using a colorimetric assay reagent according to manufacturer’s instructions (Bio-Rad). The method for ⁶⁵Zn uptake was as described for ⁶⁴Cu with the exception that HBSS uptake medium was supplemented with 0.04MBq ⁶⁵Zn (Oak Ridge Laboratory) and unlabeled ZnCl₂. HBSS was used for washes in the ⁶⁵Zn experiments was supplemented with 5 mM EDTA.

**RNA Interference**—Invitrogen Stealth™ Select RNAi 3 primer sets were used to knockdown PSEN1 (HSS108649, HSS108650, and HSS108651) and PSEN2 (HSS108652, HSS108653, and HSS108654) in HEK293T cells. All of the siRNA gene knockdown experiments included a negative control siRNA to account for any potential nonspecific gene silencing or inflammatory response (Invitrogen Stealth™ RNAi negative control LO GC). HEK293T cells were seeded in 12-well plates to be 30% confluent the following day. For each transfection 30 pmol of the appropriate siRNA and 2 µl of Lipofectamine 2000™ (Invitrogen) were diluted separately in Opti-MEM™ (Invitrogen) and mixed together after 5 min. All of the subsequent steps were carried out as per the manufacturer’s published method for transfection of siRNA (18). Knockdown efficiency was validated by Western blotting for both PSEN1 and PSEN2. Levels of PSEN1 and PSEN2 gene expression were also assessed by real time PCR.

**Inductively Coupled Plasma Mass Spectrometry Measurement of Metals**—Six-month-old male PS1+/− knock-out (n = 12) and wild type mice (n = 12) were sacrificed, and brain, liver, kidney and serum were collected and immediately frozen at −80 °C. Whole brain hemispheres and kidney and liver fragments were weighed, placed into 1.5-ml ultracentrifuge tubes, and homogenized on ice using a micropestle in 1 ml of PBS, pH 7.4, supplemented with 1X EDTA-free protease inhibitor mixture (Roche Applied Science). Homogenized tissue was centrifuged for 30 min at 100,000 × g using a Beckman Optima Max-E benchtop ultracentrifuge (Beckman Instruments). Soluble fractions were collected for protein and protein analysis, and the remaining tissue was rehomogenized in 1 ml of PBS, pH 7.4, and centrifuged for 30 min at 100,000 × g (as above). The PBS-insoluble pellet was rehomogenized and lyophilized overnight. All of the samples (triplicates) were digested in 1% HNO₃. Measurements of copper, zinc, iron, and manganese were made using a UltraMass 700 inductively coupled plasma mass spectrometry instrument (Varian Inc.) under operating conditions described previously (19).

**SOD1 Assay**—Samples were assayed for SOD1 activity using a SOD assay kit (Dojindo) according to the manufacturer’s instructions and as described previously (20). To directly measure SOD2 (MnSOD) activity, SOD1 (Cu/ZnSOD) was inhibited by the addition of 1 mM KCN and preincubated at room temperature for 5 min. The activity of SOD1 was then obtained by subtracting SOD2 activity from the total activity measured. For clarity, the data presented here are expressed as a proportion of the relevant wild type control. The samples were measured colorimetrically at 440 nm using a plate spectrophotometer (BIO-TEK Instruments).

**Statistics**—All of the analyses were performed using GraphPad Prism 5.0 (GraphPad Software). A one-way analysis of variance with Tukey’s multiple comparison test was used unless specified otherwise in the figure legends.

**Antibodies**—To detect expression levels of PS1 in the various cell lines, we used “98/1,” a polyclonal rabbit antibody raised to the PS1 N-terminal residues 1–20 as described previously (21). PS2 expression was detected using a polyclonal rabbit antibody “00/12,” raised to residues 307–336 of the human sequence PS2 loop domain as described previously (22). Polyclonal anti-SOD1 was purchased from Stressgen bioreagents. Cleavage specific anti-Notch-1 (Notch-1/Val-1744) was purchased from Cell Signaling Technology. Nicastrin was detected with a rabbit polyclonal antibody directed to residues 693–709 of human nicastrin C terminus (Sigma). Aph1a was detected using a rabbit polyclonal antibody “02/43,” raised to residues 96–110 of human sequence Aph1a as described previously (23). Presenilin enhancer 2 was detected with a rabbit polyclonal presenilin enhancer 2 antibody purchased from Abcam. APP was detected using anti-APP-CT20 (Calbiochem). Monoclonal anti-β-actin was purchased from Sigma. CCS was detected with a polyclonal rabbit antibody with an epitope corresponding to amino acids 1–274, representing full-length CCS (Santa Cruz Biotechnology).

**SDS-PAGE and Immunoblotting**—The cells were lysed in 1% Triton X-100, and 25 µg of protein from cell extracts was separated on 4–12% Bis-Tris gels (Invitrogen). The proteins were transferred to nitrocellulose for immunoblotting, and all blocking solutions, washes, and antibody dilutions were in either TBS/T or PBS/T. The signals were detected with ECL (Amer sham Biosciences), captured with the LAS-3000 imaging suite (Fujifilm), and analyzed using Multi Gauge software (Fujifilm).

**⁶⁴⁵Cu Uptake Assay**—The cells were seeded into 6- or 12-well plates and incubated at 37 °C with 5% CO₂ for 48 h prior to...
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experiment. The uptake medium was HBSS, supplemented with 1.68 M bq (or 45 μCi) of 64CaCl2 in H2O (PerkinElmer Life Sciences catalogue number NEZ013). Total calcium concentration in HBSS was 1.25 mM. The cells were incubated in uptake medium for 10 min. Uptake was stopped by washing the cell monolayers in ice-cold nonlabeled HBSS (with 2 mM EDTA) three times. The cells were then harvested with a rubber cell scraper in 0.1% SDS with 2 mM EDTA and transferred to microcentrifuge tubes. Equal volumes of scintillation fluid (Bio-Rad) were added and briefly vortexed before counting samples using a liquid scintillation counter (LKB Wallac 1211). The calcium levels were standardized to total cellular protein, which was determined for each sample using a Bio-Rad protein assay reagent according to the manufacturer’s instructions.

Real Time PCR—To measure relative PSEN1 and PSEN2 gene expression in HEK293T cells in RNAi knockdown experiments, total RNA was extracted from cells using the RNeasy kit, including DNase treatment, according to manufacturer’s instructions (Qiagen). cDNA was transcribed from 1 μg of total RNA using avian myeloblastosis virus reverse transcriptase in a 20-μl reaction according to the manufacturer’s instructions (Promega, Charbonnieres, France). Primers for real time PCR were designed using Primer3 software available on-line.

Forward and reverse primer sequences were: PS1, TCTGGTGAAAGCCAGAGGAA and AACACCGCCCAAAGGTTATG; and PS2, CTGCCAGAGAGAATGAG and CAGTGCAAGGGAGCCTAAAG, respectively. Real time PCR was performed using the Rotor Gene 3000 (Corbett Research, Mortlake, Australia). Reverse-transcribed total RNA (20 ng) was amplified in a 25-μl reaction containing 1 μM of each primer and 12.5 μl of 2× QuantiTectSYBR Green PCR Master Mix (Qiagen). The amount of gene product in each sample was determined by the comparative quantification method using the Rotor Gene 5.0 software (Corbett Research). Actin42A was used as a housekeeping gene. Forward and reverse primers for Actin42A were GCTTCCGCTGTCTACTTTCCA and CACCAGACTACTGCTTTAGA. The amount of PSEN1 and PSEN2 gene product was expressed relative to that of Actin42A to normalize for differences in total cDNA between samples.

Analysis of SOD1 Redox State in Vivo—The oxidation state of SOD1 was assessed in vivo via alkylation of reduced cysteine residues with 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) as described previously (24). For a negative control, replicate wells of attached cells were incubated in the presence of 5.2 mM iodoacetamide (Sigma) at 37 °C for 2 h to carboxymethylate cysteine residues and prevent AMS binding. Total cell extracts were prepared and preincubated on ice for 1 h with 10% trichloroacetic acid to protonate all thiols and precipitate total cellular protein. The precipitates were centrifuged at 16,000 × g for 15 min (4 °C), and the pellets were dissolved in 0.1% SDS, 0.6 M Tris-HCl, pH 8, ± 15 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (Invitrogen). The samples were incubated at 37 °C for 2 h, fractionated on 4–12% NuPage Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes, and probed with an anti-SOD1 polyclonal antibody.

Alkaline Phosphatase (ALP) Activity Assay—MEF cells (∼2 × 106) were collected, washed in PBS, and solubilized in ALP lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl2, and 0.1% Triton X-100). Total cellular protein concentration was determined using a Bio-Rad (Bradford) protein assay. Using a method adapted from Suzuki et al. (25), 60 μg of each sample was preincubated in 96-well microtitre plates for 10 min at room temperature. A 200-μl substrate solution (2 mg/ml p-nitrophphenyl phosphate in 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM MgCl2) was added. The release of p-nitrophensol was measured by the absorbance at 405 nm. The measurements were taken constantly over a 30-min period at 37 °C. Shrimp ALP (Sigma) was used as a standard.

RESULTS

Presenilin Is Required for Rapid Copper Uptake—To study the contribution of PS to copper uptake, we used cultured MEFs derived from PS1−/−, PS2−/−, and PS1/PS2 double knock-out (PS dKO) and WT embryos (15). We found that the initial rate of 64Cu uptake was markedly reduced in MEF cells that are PS1- and/or PS2-deficient (Fig. 1A and supplemental Fig. S1A). 64Cu uptake in WT MEFs was saturable across a range of copper concentrations at 37 °C and fitted Michaelis-Menten kinetics ($K_m = 7.0 ± 1.3$ (S.E.), $V_{max} = 18.2 ± 1.2$ pmol/mg protein/min) (Fig. 1A). In PS dKO MEFs, the rate of 64Cu uptake was much lower and saturated at a lower copper concentration than WT MEFs ($K_m = 2.5 ± 0.8$ (S.E.), $V_{max} = 6.4 ± 0.5$ pmol/mg protein/min), indicating a copper uptake deficit in the absence of PS expression (Fig. 1A). Cooling to 4 °C abolished saturable uptake of 64Cu in both WT and PS dKO MEFs, and there was no significant difference between the two cell lines in this nonspecific uptake (Fig. 1A, dashed lines). Therefore, the contribution of cellular copper import that is PS-mediated may be energy-dependent. We subtracted the 4 °C data from the 37 °C data to obtain the apparent $K_m$ and $V_{max}$ values. We then subtracted the initial velocity ($V_i$) values of PS dKO MEFs from WT MEFs and recalculated the Michaelis-Menten kinetic parameters attributable to PS-mediated 64Cu2+ uptake, which revealed saturability at approximately the copper concentration range of the glutamatergic synapse (26) ($K_m = 14.9 ± 5.2$ μM and $V_{max} = 13.3 ± 2.0$ pmol/mg protein/min). Both PS1 and PS2 were required for normal copper uptake because knock-out of either gene significantly inhibited the accumulation of cellular 64Cu over a period of 4 h (Fig. 1B). The reduction in time-dependent copper accumulation was greater in PS1 knock-out cells than PS2 knock-out cells, and the reduction in copper accumulation in MEF cells that lack both PS1 and PS2 was similar to that of PS1 knock-out alone, suggesting that PS1-mediated copper uptake may be more critical than PS2 (Fig. 1B).

To address whether altered copper status in PS dKO MEFs could be due to an increase in cellular copper efflux, we performed pulse-chase studies. Initial rates of copper efflux are difficult to measure because of large errors associated with low radioactive counts. We measured copper retention over a longer period (2 h), which provides a surrogate estimate of efflux (27). Knock-out of PS did not decrease cellular 64Cu retention, expressed as a proportion of the amount taken up at 2 h (Fig. 1C). Therefore, PSs principally affect uptake of copper rather than efflux.
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To rescue the copper uptake deficiency in PS dKO cells, we used cells that had been virally transduced with human presenilin 1 (hPS1) (15). Expression of hPS1 was partially able to restore copper uptake, although not to the same level as control (WT) cells (Fig. 1D). Expression of hPS1 in PS dKO cells was sufficient to restore APP processing and expression of the γ-secretase subunits, nicastrin, and Pen-2 (supplemental Fig. S2A). However, processing of another γ-secretase substrate, Notch-1, and expression of Aph1a, a subunit of γ-secretase, was not fully restored (supplemental Fig. S2B). Expression of the hPS1 was greater than the expression of endogenous mouse PS1 in WT cells. Therefore, incomplete restoration of copper uptake could not be explained by insufficient PS1 expression (supplemental Fig. S2B). Why expression of hPS1 in a PS null mouse background can fully restore some PS activities but not others is not clear. There is a large degree of heterogeneity in γ-secretase complexes (28), and full restoration of PS-dependent activities could reflect the balance of expression between γ-secretase components, including PS2 and Aph1a.

Expression of a catalytic mutant construct, D385A hPS1, which lacks γ-secretase function (15), also partially restored copper uptake in PS dKO cells. Copper uptake was greater than for WT hPS1 transduced cells, indicating that the effect of PS on copper uptake may be independent of γ-secretase activity (Fig. 1D).

Presenilin Is Required for Rapid Zinc Uptake—We investigated whether PS expression may promote the uptake of Zn^{2+}. The initial rate of ^{65}Zn uptake was reduced in PS knock-out MEFs compared with WT MEFs (Fig. 2A and supplemental Fig. S1B). In both WT and PS dKO MEFs, initial Zn^{2+} uptake was saturable and fitted Michaelis-Menten kinetics. WT MEFs had an apparent $K_m$ of 184.4 ± 103.4 μM Zn^{2+} and a $V_{max}$ of 3044 ± 776.1 pmol/mg protein/min, whereas PS dKO MEFs had an apparent $K_m$ of 126.4 ± 72.8 μM Zn^{2+} and a $V_{max}$ of 1022 ± 237.4 pmol/mg protein/min (Fig. 2A). To determine the contribution of PS to zinc uptake, we subtracted the initial velocity of Zn^{2+} uptake in PS dKO MEFs from WT MEFs to estimate the Michaelis-Menten kinetic parameters of $K_m$ =...
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231.4 ± 207.3 μM and $V_{\text{max}} = 2060 ± 901.3$ pmol/mg protein/min. The $K_m$ values for Zn$^{2+}$ uptake, although much higher than for Cu$^{2+}$, are within a physiological range consistent with synaptic zinc concentrations (11). Although the calculated $K_m$ values for zinc uptake did not differ significantly between normal and PS null cells, the maximal velocity of zinc uptake was significantly less in the PS dKO cells. This can be interpreted in one of two ways. Either fewer zinc transporters are available on the plasma membrane for zinc import, or the passage of zinc via plasma membrane zinc transporters is impaired by the loss of PS. Upon cooling to 4 °C, saturable $65$Zn uptake was abolished, as with $64$Cu uptake (Fig. 1A), which may reflect nonspecific surface binding of zinc, and there was no significant difference between WT and PS dKO MEFs (Fig. 2A). Even at a low exogenous zinc concentration of 2.5 μM, time-dependent $65$Zn accumulation was significantly reduced in PS1 KO, PS2 KO, and PS dKO MEFs compared with WT MEFs (Fig. 2A).

We performed a rapid pulse-chase experiment for $65$Zn to determine whether PS deficiency affects Zn$^{2+}$ retention. Cellular Zn$^{2+}$ retention, expressed as a proportion of cellular $65$Zn taken up after 10 min, was not decreased in PS dKO MEFs compared with WT MEFs following a 30-s pulse-chase (Fig. 2C). This indicates that low Zn$^{2+}$ retention in PS null cells is not likely to be due to facilitated export. Expression of WT human PS1, but not mutant D385A hPS1, in mouse PS dKO MEFs enabled partial restoration of Zn$^{2+}$ uptake (Fig. 2D). The rescue of Zn$^{2+}$ uptake by expression of hPS1 was not complete and could be consistent with the need for both PS1 and PS2 to be expressed for optimal zinc uptake. The inability of the catalytically inactive D385A hPS1 to rescue Zn$^{2+}$ uptake could not be explained by the lack of expression, although the mutant protein was clearly not processed (supplemental Fig. S2B).

**SOD1 Activity Is Reduced in PS-deficient Cultured MEF Cells**—To determine whether the loss of PS impacts upon intracellular copper and zinc biochemical targets, we analyzed the enzyme Cu/ZnSOD1 that requires both Cu$^{2+}$ and Zn$^{2+}$ for its activity. There was a marked and significant reduction (~95%) in the specific activity of SOD1 in PS dKO MEF cells compared with WT MEF cells (Fig. 3A), consistent with impaired metallation of SOD1. A possible explanation for impaired SOD1 activity is...
oxidative modification to critical cysteine residues that then prevents correct disulfide bond formation (29). To investigate this, we used AMS, a compound that binds to “free” or reduced cysteine thiol groups, causing an electrophoretic mobility shift. We did not detect any difference in AMS binding in PS dKO or WT MEFs, evidenced by similar SOD1 electrophoretic patterns following AMS treatment (supplemental Fig. S3). Knock-out of either PS1 or PS2 alone was insufficient to lower SOD1 specific activity (Fig. 3A). Expression of hPS1 in a PS dKO background restored SOD1 specific activity to normal WT level, and expression of D385A mutant hPS1 led to an increase in SOD1 activity greater than that of nontransduced WT cells (Fig. 3A), partly consistent with the superior ability of the mutant to promote cellular copper import (Fig. 1D). It is possible that the D385A mutant hPS1 favors the loading of copper into SOD1 over other intracellular cuproproteins. Presenilin ablation did not affect SOD1 protein levels in cultured MEF cells (Fig. 3B), indicating that a loss of SOD1 activity in PS null MEFs is not simply due to reduced SOD1 expression. We also measured the activity of ALP, a well characterized zinc-dependent enzyme (25, 30). In contrast to SOD1, the activity of ALP was inhibited, and indeed PS-depleted MEFs lost significantly less copper and zinc in the PBS-insoluble fractions from kidney of PS dKO mice (Fig. 5). Therefore, copper and zinc export must be inhibited, and indeed PS-depleted MEFs lost significantly less Cu and Zn over a 2-h washout than WT cells (Figs. 1C and 2C). Hence, the turnover of copper and zinc is impaired in PS-deficient MEFs. This led us to suspect that copper or zinc may not be loaded correctly into SOD1 in cells that lack PS1. To assess this, we measured expression of the copper chaperone for SOD1, CCS, and found that it was greatly reduced in PS-deficient MEFs (Fig. 4). This is a likely explanation for why such a severe loss of SOD1 activity is observed in PS-deficient cells. Expression of hPS1 in PS dKO MEFs partially restored CCS expression (Fig. 4). Although the elevated CCS in hPS1-transduced PS dKO MEFs was accompanied by restoration of SOD1 activity (Fig. 3A), the SOD1 activity now surmounted baseline activity. The explanation for this overshoot in SOD1 activity is unclear, but possibly human PS1 has enhanced effects on this pathway compared with mouse PS.

**PS1 Knock-out Mice Have Tissue-specific Copper and Zinc Deficiencies**—Measurement of total tissue copper and zinc in 6-month-old PS1+/+ hemizygote knock-out mice (16) revealed tissue-specific differences when compared with age-matched wild type littermate controls (PS1+/−). Copper, iron, zinc, and manganese levels in brain, liver, kidney, and serum were measured using inductively coupled plasma mass spectrometry. The tissues were homogenized in PBS and centrifuged at 100,000 × g to separate soluble and pellet fractions. We found significantly less brain copper (−15%) and zinc (−13%) in the soluble fractions of PS1−/− mice compared with PS1+/− mice (Fig. 5, A and B), consistent with a steady-state copper and zinc deficiency in the brain. There were significant decreases in both copper and zinc in the PBS-insoluble fractions from kidney of PS1−/− mice versus PS1+/− mice (Fig. 5, C and D). There were no differences in liver copper between PS1−/− and PS1+/− mice (Fig. 5E), but liver zinc was lower in the soluble fractions of...
PS1+/− mice (Fig. 5F). No significant differences between PS1 genotypes were detected for either copper or zinc in serum (Fig. 5G), and there were no differences for iron or manganese in any of the tissues sampled, except for an increase in iron in the soluble fraction of kidney homogenate (supplemental Fig. S6).

**Lowered Brain SOD1 Activity and CCS Levels in PS1+/− Mice**—SOD1 specific activity was significantly reduced (approximately −15%) in the soluble fraction of brain lysates from 6-month-old PS1+/− mice compared with PS1+/+ mice (Fig. 6A), consistent with our observation of reduced SOD1 activity in cultured PS knock-out MEFs (Fig. 3B). CCS levels in the PS1+/− brain tissue were significantly decreased by about the same proportion as SOD1 specific activity (Fig. 6B), in accord with the changes in the PS dKO MEFs (Fig. 4). SOD2 activity was unaltered in both the PS knock-out MEFs and the brains of PS1+/− mice, compared with WT controls (supplemental Fig. S7).

**RNA Interference of PSEN Expression Reduces Copper and Zinc Uptake in HEK293T Cells**—To confirm a role for PS in metal uptake in an alternative cell line, we performed targeted RNA interference of PS1 and PS2 expression in cultured human embryonic kidney (HEK293T) cells, which express significant levels of PS1, PS2, and APP. In concordance with the results for MEF cells (Fig. 1), RNA interference of endogenous PS1 or PS2 expression in HEK293T cells caused a significant reduction in [C]Cu uptake (Fig. 7A) and [Zn]Zn accumulation compared with nontreated cells or cells treated with scrambled siRNA. Western blotting of samples harvested from parallel cultures assessed expression of both PS1 and PS2 proteins; greater than 75% knockdown of endogenous PS1 or PS2 expression was achieved using specific siRNA compared with nontreated or scrambled siRNA-treated HEK293T cells (Fig. 7C). PSEN1 and PSEN2 RNA was similarly reduced (supplemental Fig. S8). The inhibition of copper and zinc uptake was proportionally less in the RNAi experiments than in the studies of MEF cells (Fig. 1). This is possibly because the RNAi knockdown did not totally abolish PS expression or because HEK cells rely less upon PS for copper and zinc uptake than MEFs, instead using alternative uptake pathways.

**Presenilin Mediates Copper and Zinc Import Independently of Its γ-Secretase Activity**—We examined whether pharmacological inhibition of γ-secretase activity could modulate Cu2+ or Zn2+ import. The γ-secretase inhibitor, DAPT, did not alter Cu2+ or Zn2+ import in HEK293T cells compared with vehicle-treated control cells (Fig. 8, A and B, respectively), despite causing a dose-dependent accumulation of APP C-terminal fragments (Fig. 8C). Similarly, copper uptake was not significantly altered by DAPT treatment in MEFs or several other cell lines, including M17 human neuroblastoma or N2a mouse neuroblastoma (Fig. 8D). Combined with the ability of expressed γ-secretase catalytic mutant, D385A hPS1, to partially restore Cu2+ uptake in PS dKO MEF cells (Fig. 1D), lack of an effect with DAPT in multiple cell types indicates that copper uptake mediated by PS does not depend on γ-secretase activity.
Presenilin-mediated Copper and Zinc Cell Influx Does Not Occur via Calcium Channels—PS has been shown to modulate intracellular calcium stores (31–35), so it was important to establish whether the PS-mediated uptake of copper and zinc could occur via calcium channel activity. We reasoned that if extracellular calcium uptake was affected by a loss of PS, then copper and zinc entry via these channels is plausible. We performed 45Ca uptakes using conditions similar to those used for 64Cu and 65Zn uptake assays but observed no change in calcium uptake in PS-deficient MEF cells or PS RNAi-treated HEK293T cells at physiological calcium concentrations (supplemental Fig. S9). Similarly, thapsigargin, a specific inhibitor of the ER Ca-ATPase pump (36) that controls capacitative calcium entry at the plasma membrane, did not significantly affect copper or zinc uptake in cultured WT or PS dKO MEF cells (supplemental Fig. S10). In light of these data, it is unlikely that promiscuous entry of copper and/or zinc via plasma membrane-localized calcium channels can account for the significant difference in uptake of these metals between WT and PS dKO MEF cells.

DISCUSSION

Our data reveal that PS1 and PS2 play significant roles in the cellular uptake of copper and zinc, highlighted by reduced levels of both of these metals in the brains of PS1-deficient mice. Because the uptake rate for both metal ions is decreased by ~50% in MEFs (Figs. 1 and 2) but steady-state metal levels are unchanged, we deduce that total cellular turnover of copper and zinc is slowed by the absence of PS.

We also found that the specific activity of SOD1 was sensitive to PS deficiency in MEFs (Fig. 3) and mouse brain (Fig. 6). This could not be a nonspecific consequence of reduced copper and/or zinc cellular uptake leading to steady-state metal deficiency in the PS-null MEFs because basal levels of both of these metals were unchanged and because zinc-dependent ALP activity was undiminished. Modification of SOD1 cysteine side chains was excluded as an explanation for decreased SOD1 activity in the PS-null MEFs (supplemental Fig. S3).

The decrease in SOD1 activity in PS-null MEFs and PS1/−/− brain tissue was associated with decreased protein expression of CCS, which facilitates the loading of copper into the active site of SOD1 (37). Hence, presenilin appears to influence not only copper uptake but also its intracellular distribution toward an important antioxidant system. Whether this interaction has any importance for pathophysiology remains to be explored, but notably, relevant adverse effects in familial Alzheimer disease-linked PS mutant models have been reported: PS1M146V knock-in mice have increased oxygen radical production (38), and PS1M146L knock-in mice have decreased brain SOD1 activity (39). In light of our findings, we hypothesize that some of the reported effects of PS dysfunction are consequences of deficient copper and/or zinc turnover and distribution.

The impact of PS on CCS expression was surprising because copper deficiency has been described to increase CCS expression (40, 41). Our findings therefore implicate PS in the expression or stability of CCS by a mechanism that is still uncertain.
Presenilin Mediates Copper and Zinc Uptake

The mechanism underlying our observations of decreased Cu\(^{2+}\) and Zn\(^{2+}\) intake in PS-null cells and lowered copper and zinc levels in PS1 hemizygous mice could be a downstream effect of altered protein trafficking. PS has been shown to affect membrane protein trafficking from the ER via its \(\gamma\)-secretase activity (47), and several of these substrates and products, including APP and A\(\beta\), bind copper and zinc (47–50). PS deficiency also results in the mislocalization of the copper-dependent enzyme tyrosinase and the accumulation of tyrosinase C-terminal fragments in cultured mouse melanocytes and retinal pigment epithelial cells (51). Such findings could be consistent with a role for PS in copper and zinc homeostasis via protein trafficking. Although many aspects of the uptake process remain uncertain, evidence suggests that both Cu\(^{2+}\) and Zn\(^{2+}\) enter cells by binding to copper transporter 1 or a ZIP on the surface and are then ferried within an endosome. Notably, like CCS, ZIP1 also rescued the dominant negative Psn phenotype in Drosophila (42). We hypothesize that PS is involved in handling this metal intake endosome through autophagic pathways that have recently come to light (52).

A direct role for PS1 in autophagy came into focus recently with evidence that PS1 holoproteins are required for N-glycosylation and subsequent maturation and trafficking of the vATPase responsible for lysosome acidification (52). This has important implications for considering the mechanism underlying PS-facilitated metal uptake because the divalent metal transporter 1 is a proton-dependent cation pump that relies on vATPase-dependent acidification (53). Intriguingly, a vATPase, Vha55 was also on the list of genes that could rescue the dominant negative Psn phenotype in Drosophila (42). Divalent metal transporter 1 mediates the cellular import of nonheme Fe\(^{3+}\) but also transports other divalent metal ions, including Cu\(^{2+}\) and Zn\(^{2+}\) (54). A functional link between PS-mediated vATPase activation and divalent metal transporter 1-mediated ion transport is currently being investigated.

Our findings also indicate that the PS-mediated metal intake mechanism is independent of \(\gamma\)-secretase activity: the \(\gamma\)-secretase inhibitor DAPT had no effect on Cu\(^{2+}\) or Zn\(^{2+}\) uptake (Fig. 7), and the \(\gamma\)-secretase inactive mutant D385A hPS1 was more efficient at restoring copper uptake (Fig. 1D) and SOD1 activity (Fig. 3) than wild type hPS1 in knock-out cells that lack endogenous PS. This could be consistent with PS influencing the trafficking of (as yet unidentified) metal-transport proteins that are not \(\gamma\)-secretase substrates. Notable examples of proteins that are not \(\gamma\)-secretase substrates yet accumulate in autophagic organelles in PS1-deficient neurons include ICAM-5 (telencephalin) (55) and \(\alpha\)- and \(\beta\)-synucleins (56). Also notable, expression of the D385A mutant PS1 in our studies was less efficient at restoring zinc uptake than wild type hPS1 in the PS dKO MEF cells (Fig. 2D), indicating that the mutation might selectively disadvantage Zn\(^{2+}\) uptake.

Although there are multiple ZIPS, copper transporter 1 is the only plasma membrane-located Cu\(^{1+}\) import protein in mammalian cells (57). Our current findings may help explain an alternative copper import mechanism that has been described previously. The Michaelis-Menten kinetic values for copper uptake reported in the literature range vary from \(K_m = 1.71 \mu M\) and \(V_{max} = 6.76 \text{ pmol}\)}
copper/min/mg protein in HEK293 cells to $K_m = 8.9 \mu M$ and $V_{max} = 75.7$ pmol copper/min/mg protein in Sf9 cells, with variation attributable to such factors as cell type and experimental conditions (5, 58). However, copper transporter 1 null MEF cells exhibited residual copper uptake of ~30%, and an unknown mammalian Cu$^{2+}$ import mechanism with a $K_m$ of ~10 $\mu M$ has been predicted (5). Consistent with this, PS mediates Cu$^{2+}$ uptake with an apparent $K_m$ of 14.9 $\mu M$.

Aβ selectively binds Cu$^{2+}$ and Zn$^{2+}$, which promote oligomerization and precipitation (48, 50). Strong evidence implicates hypermetallation of Aβ by copper and zinc in the synaptic vicinity as driving amyloid pathology (14, 59, 60). Our current findings indicate that PS has the capacity to influence extraneuronal copper and zinc concentrations. In glutamatergic synapses, where transient levels of Zn$^{2+}$ and Cu$^{2+}$ are uniquely high, the loss of PS function, either by reduced expression or mutation, may allow extracellular copper and zinc to pool at the region where Aβ concentrations are highest (61) and prone to metal-induced aggregation. We are currently investigating the role of familial Alzheimer disease mutations further to see whether they alter zinc or copper uptake in a manner that could promote Aβ oligomerization.

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