Selenoprotein P (SELENOP1) is a selenium-rich antioxidant protein involved in extracellular transport of selenium (Se). SELENOP1 also has metal binding properties. The trace element Zinc (Zn\(^{2+}\)) is a neuromodulator that can be released from synaptic terminals in the brain, primarily from a subset of glutamatergic terminals. Both Zn\(^{2+}\) and Se are necessary for normal brain function. Although these ions can bind together with high affinity, the biological significance of an interaction of SELENOP1 with Zn\(^{2+}\) has not been investigated. We examined changes in brain Zn\(^{2+}\) in SELENOP1 knockout (KO) animals. Timm-Danscher and N-(6-methoxy-8-quinolyl)-p-toluenesulphonamide (TSQ) staining revealed increased levels of intracellular Zn\(^{2+}\) in the SELENOP1\(^{-/-}\) hippocampus compared to wildtype (WT) mice. Mass spectrometry analysis of frozen whole brain samples demonstrated that total Zn\(^{2+}\) was not increased in the SELENOP1\(^{-/-}\) mice, suggesting only local changes in Zn\(^{2+}\) distribution. Unexpectedly, live Zn\(^{2+}\) imaging of hippocampal slices with a selective extracellular fluorescent Zn\(^{2+}\) indicator (FluoZin-3) showed that SELENOP1\(^{-/-}\) mice have impaired Zn\(^{2+}\) release in response to KCl-induced neuron depolarization. The zinc/metal storage protein metallothionein 3 (MT-3) was increased in SELENOP1\(^{-/-}\) hippocampus relative to wildtype, possibly in response to an elevated Zn\(^{2+}\) content. We found that depriving cultured cells of selenium resulted in increased intracellular Zn\(^{2+}\), as did inhibition of selenoprotein GPX4 but not GPX1, suggesting the increased Zn\(^{2+}\) in SELENOP1\(^{-/-}\) mice is due to a downregulation of antioxidant selenoproteins and subsequent release of Zn\(^{2+}\) from intracellular stores. Surprisingly, we found increased tau phosphorylation in the hippocampus of SELENOP1\(^{-/-}\) mice, possibly resulting from intracellular zinc changes. Our findings reveal important roles for SELENOP1 in the maintenance of synaptic Zn\(^{2+}\) physiology and preventing tau hyperphosphorylation.

Keywords: Selenoprotein P, zinc, Alzheimer's disease, tau, selenium
INTRODUCTION

Within the body, selenium (Se) functions primarily in the form of selenocysteine (Sec), the 21st amino acid, which is incorporated into members of the selenoprotein family (1–3). Selenoprotein P (SELENOP1) is a selenium-rich protein with 10 Sec residues that transports Se in serum from liver to the brain and other organs (4). SELENOP1 is present in the cerebral spinal fluid (CSF) and in the choroid plexus, which releases CSF (5, 6), and in glial cells (7). SELENOP1 has also been described in brain neurons (8, 9), which may be the targets of Se transport. SELENOP1 KO mice have reduced brain selenium and reduced levels of antioxidant selenoproteins such as glutathione peroxidases 1 and 4 (GPX1 and GPX4) (10). Mice with the SELENOP1 gene deletion have deficient hippocampal synaptic function and deficits in spatial learning and long-term potentiation (LTP), a model for learning and memory (11). SELENOP1 is increased in the brain and CSF in Alzheimer’s disease (5, 8, 12) and associated with both Alzheimer’s and Parkinson’s pathology (8, 13).

Se and Zn²⁺ are both essential trace elements required for proper brain function. Selenium deficiency correlates with impaired cognitive and motor function (14, 15), while Zn²⁺ deficiency correlates with decreased nerve conduction and impaired cognitive performance (16). Alzheimer’s disease is associated with increased brain Zn²⁺ levels (17). Zn²⁺ can increase tau phosphorylation (18, 19), which contributes to the formation of neurofibrillary tangles, a hallmark of Alzheimer’s disease (20). However, studies have yet to address the biological relevance of the interaction between these elements despite their high affinity for each other and their importance in brain function.

Figure 1A shows SELENOP1’s two functional, glycosylated domains: (1) a Se-rich C-terminal domain with 9 S residues, (2) an N-terminal domain with 1 S (U) in U-x-x-C redox motif, 2 histidine-rich metal binding sites (located at residue 204–217 and residue 244–250) and a heparin binding site (4, 21). SELENOP1 also has an N-terminal signal peptide for extracellular secretion, which is cleaved in the Golgi (22).

We hypothesized that SELENOP1, as a metal-binding protein, could have a role in brain Zn²⁺ homeostasis. In this study, we investigated whether SELENOP1 influences brain Zn²⁺ by evaluating changes in hippocampal Zn²⁺ in SELENOP1 KO animals. Here we report that deletion of SELENOP1 alters levels of chelatable Zn²⁺ and prevents the release of synaptic Zn²⁺ in mouse hippocampus. These findings indicate an important additional role for SELENOP1 in the regulation of zinc in the brain. They could also have important implications for the treatment of disorders where Zn²⁺ physiology is impaired.

MATERIALS AND METHODS

Animals
Mice were group housed on a 12-h light cycle and provided food and water ad libitum. All animals in this study were maintained on diets containing adequate selenium (~0.25 ppm) and zinc (~80 ppm). All mice used were 3–6 months of age and included both male and female mice as indicated. All animal procedures were approved by the University of Hawaii Institutional Animal Care and Use Committee.

SELENOP1⁻/⁻ mice were obtained from the laboratory of Dr. Raymond Burk at Vanderbilt University. The mutant mice were backcrossed to C57BL/6J for at least ten generations with C57BL/6J mice from Jackson Laboratories to ensure congenic strains. Breeding of SELENOP1⁺/⁻ mice generated littermates of SELENOP1⁺/+ and SELENOP1⁻/⁻ pups, which were used in this study in addition to SELENOP1⁺/⁻ mice. Genomic DNA extracted from mice tails was used for genotyping PCR using specific primers (forward-ACCTCAGC AATGTGGAGAAGCC, reverse-TGCCCTCTGAGTTTAGC ATTGTGGAGAAGCC, reverse-TGCCCTCTGAGTTTAGC ATTGTGGAGAAGCC, reverse-GATGATCTGGACG AAGAGCATCA for SELENOP1⁻/⁻). Products were run on a 1.5% DNA agarose gel with a SYBR Safe DNA gel stain (Invitrogen) and genotypes were confirmed under UV light.

Timm-Danscher Zn²⁺ Labeling
The Danscher modification of Timm’s zinc stain (“neo-Timm’s”) was used to label intracellular chelatable zinc (i.e., not tightly bound to proteins or other molecules) (23). Deeply anesthetized mice received intraperitoneal (IP) injections of 20 mg/kg sodium selenite (2 mg/ml in normal saline). After 2 h, mice were perfused with saline followed by 4% paraformaldehyde (PFA) in saline. Brains were postfixed overnight in 4% PFA, dehydrated serially with PBS, sections were developed with the IntenSE M silver Enhancement kit (Amersham International) according to the manufacturer’s protocol and as previously described (24).

6-Methoxy-8-P-Toluenesulfonamido-Quinoline (TSQ) Stain
TSQ is a Zn²⁺ fluorophore that binds intracellular chelatable Zn²⁺ in a 2:1 ligand-to-metal ratio that results in increased fluorescence emission at 490 nm in response to excitation at 360 nm (25). Serial sagittal cryosections (10 µm) of brain hemispheres were mounted on positively charged microscope slides. The slides were immersed with 4.5 µM TSQ (Enzo Lifesciences, UltraPure) in 140 mM sodium barbital and 140 mM sodium acetate buffer (pH 10) for 90 s, as previously described (26) and washed in 0.1% NaCl. TSQ-stained sections were imaged using DAPI filter settings (200 ms, monochrome with a 5x objective). The mean fluorescence intensity of the hippocampal CA1 stratum oriens and stratum radiatum, CA3 mossy fibers, and hilar region were measured with ImageJ software (NIH). The background was measured in unstained areas within lateral ventricles and subtracted from mean TSQ signals.
FIGURE 1 | Zinc-binding properties of SELENOP1. (A) Schematic of SELENOP1 structure. The N-terminal region has 1 S (U) in a redox domain, a heparin-binding site and a zinc transporter domain with two metal-binding sites. The C-terminal region has 9 S residues for Se transport. Sites of residues predicted to bind zinc using Pred zinc are indicated by *. (B) Alignment of human SELENOP1 metal binding region with other proteins containing the zincin motif. (C) Western blot of metal column eluates after applying mouse serum from WT (+/+) or SELENOP1 KO (−/−) mice to mini-columns with agarose only or agarose bound to Co^{2+}, Ni^{2+}, and Zn^{2+}. Untreated SELENOP1+/− and WT serum were also added to the blot as a positive control (left lanes). SELENOP1 protein detected with anti-SELENOP1 antibody (1:1000) had a molecular weight of approximately 55 kDa in the wildtype serum, which was not seen in SELENOP1−/− serum. SELENOP1 was detected in column eluates from wild-type serum applied to all metal columns, but not from columns with agarose only.

Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)
The metal concentrations within frozen right brain hemispheres and liver samples were processed at the Agricultural Diagnostic Service Center (ADSC) run by the College of Tropical Agriculture and Human Resources (CTAHR), University of Hawaii. Dry ash sample preparations were subjected to acid digest before ICP-OES (0.01 ppm detection limit) to measure total brain and liver metal content (Zn^{2+}, Cu^{2+}, Fe^{2+}). Water blanks and solution standards were included with each run to calibrate results.

Protein Extraction and Western Blot
Proteins were extracted from frozen hippocampal tissue using CelLytic MT buffer (Sigma) per the manufacturer’s instructions, denatured by heating in Laemmli sample buffer, resolved by SDS-PAGE on a 10–20% gradient Tris-HCl Criterion Precast gel (Bio-Rad Laboratories), and electrically transferred to polyvinylidene difluoride (PVDF) membranes. For detection of
Zn\(^{2+}\) regulating proteins, membranes were incubated in anti-
metallothionein-3 (1:500, rabbit polyclonal, BioSent), and anti-
ZnT1 and anti-ZnT3 (1:1000; 1:5000, rabbit polyclonal, Synaptic
Systems). For detection of the SELENOP1 protein, membranes
were incubated in anti-SELENOP1 (1:1000, rabbit monoclonal,
Proteintech). For measurement of tau, antibodies recognizing tau
phosphorylated at T231, S214 or S396 (1:1000, Invitrogen) or t au
were incubated in anti-SELENOP1 (1:1000, rabbit monoclonal,
Novus Biologicals) to control for loading. Membranes were imaged with the Odyssey infrared
fluorescence system (LiCor), and densitometry analysis was
performed on the ImageStudio software (LiCor).

**Metal Agarose Column Purification**

To observe SELENOP1 binding to biometals, we used mini
spin-columns containing high-density agarose beads conjugated with Zn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) (Agarose Bead Technologies) to isolate metal-binding proteins from wildtype and SELENOP1\(^{-/-}\)
mouse serum. A metal-free agarose column served as a negative
control. Serum samples were diluted 1:100 in PBS and added
to the column, gently shaken for 60 min at 4°C, then spun
for 60 s at 800x g to collect flow-through. Columns were washed with increasing concentrations of imidazole (0, 10,
and 20 mM) diluted in PBS, and then bound proteins were eluted with 250 mM imidazole diluted in PBS. Eluted proteins
were determined with western blot using an anti-SELENOP1 antibody (27).

**Live Hippocampal Slice Imaging**

To measure stimulus-induced extracellular Zn\(^{2+}\) accumulation, hippocampal slices were prepared from 3 to 6 month old
SELENOP1 KO and wild-type littermate mice as previously
described (28). Following slice preparation, slices were
acclimated to room temperature and superfused with oxygenated
(95 O\(_2\) and 5% CO\(_2\) gas mix) artificial cerebral spinal fluid (ACSF,
composition in mM: NaCl 130; KCl 3.5; glucose 10; NaHCO\(_3\)
24; NaH\(_2\)PO\(_4\) 1.25; MgSO\(_4\) 1.5; CaCl\(_2\) 2.0) for at least 60 min.
The CA1 stratum radiatum region of the slices was imaged with a ZEISS laser-scanning microscope using a 10X objective with
the pinhole fully opened at 1 frame/s at 640 × 480 resolution.
Cell-impermeant FluoZin-3 at 1.5 µM (Molecular Probes) was added to the ACSF to detect extracellular Zn\(^{2+}\) accumulation from hippocampal slices in response to the administration of a depolarizing (35 mM) KCl concentration for 60 s. In some experiments, a slow onset Zn\(^{2+}\) chelator (Ca\(^{2+}\)-EDTA) was added to remove contaminating Zn\(^{2+}\) in media and to reduce background fluorescence (29, 30). Fluorescence intensities of hippocampal slices in the CA1 stratum radiatum region upon addition of KCl for each slice were expressed as the fluorescence intensity over the fluorescence during baseline (F/F\(_0\)). The mean area under the curve during application of high K\(^{+}\) for signals of SELENOP1\(^{-/-}\) slices were compared to WT to determine if changes in Zn\(^{2+}\) release were altered.

**FluoZin-3 Measurements in Cell Culture**

SH-SY5Y cells were plated in 96-well plates and differentiated by exposure to Neurobasal media (Invitrogen) supplemented with B27 (Invitrogen) for 48 hrs. The media was then changed to Roth-Schweizer media (31) but with of 0, 10 or 100 nM. Alternatively, cultures in 10 nM Se were treated with 100 µM mercaptosuccinate (MCS), 0.1 µM RSL-3, 0.1 µM RSL-3 +
100 µM α-tocopherol, or 0.01 µM DMSO as a control for RSL-3.
Cells were treated for either 5 days (DMSO, RSL-3, RSL-3 with α-tocopherol) or 7 days (Se concentrations and remaining conditions). Cells were rinsed twice in HEPES buffered saline
(HBSS: (in mM) NaCl 146; KCl 3.5; glucose 10; 1.25; MgSO\(_4\)
1.5; CaCl\(_2\) 2.0, HEPES 10, NaOH 10]. Cells were loaded with 1 µM FluoZin-3 AM (Thermo Fisher Scientific) with 0.02% pluoronic F-127 for 30 min at 37°C in the dark, then rinsed
twice with HBSS, and the fluorescence measured in HBSS.
For additional controls, either 100 µM 2, 2-dithiodipyridine
(DTDP), 100 µM tetrakis-(2-pyridylmethyl) ethylenediamine
(TPEN), or 100 µM H\(_2\)O\(_2\) were added to the HBSS 10 min before measuring fluorescence. Plates were scanned in a SpectraMax M3 fluorescent plate reader (Molecular Devices) with 494 nm excitation, 516 nm emission. After scanning, cells were fixed in 100% methanol at −20°C overnight, stained with 100 µg propidium iodide (PI), rinsed twice with HBSS, and then scanned
in HBSS at 536 nm excitation, 617 nm emission. FluoZin-3 fluorescence was normalized to PI fluorescence to correct for any differences in cell density.

**Statistical Analysis**

All statistical analyses were carried out with Graphpad Prism
Software with measurements given as means ± SE. Comparisons between treatments, genotypes and sex were performed by student’s unpaired t-test and one-way or two-way analysis of variance (ANOVA), with p < 0.05 considered significant. In general, we did not find sex differences in Zn\(^{2+}\) distribution or release unless shown; otherwise, males and females were averaged together by genotype.

**RESULTS**

**Zn\(^{2+}\) Binding Properties of SELENOP1**

Previous studies described the affinity of SELENOP1 for several metals, including Zn\(^{2+}\). However, the biological interaction of SELENOP1 with Zn\(^{2+}\) is unclear. We investigated potential Zn\(^{2+}\)-binding domains of SELENOP1. Using the web-based software Predzinc (https://predzinc.bioshu.se/pred/), a web server that predicts zinc-binding proteins and zinc-binding sites from given sequences, we analyzed the SELENOP1 coding sequences for potential zinc-binding sites. We found that a His residue within the SELENOP1 His-rich metal binding domain as well as two other His residues in the C-terminal region are predicted to be Zn\(^{2+}\) binding motifs, shown in Figure 1A by asterisks (*).

A domain search of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database indicates that the human SELENOP1 protein structure has a zinc-binding domain overlapping one of the His-rich regions, which has homology to the ZIP zinc
transporter domain. Additionally, within this sequence is a zincin 
\( \text{Zn}^{2+} \)-binding motif, generally found in the metzincin family of 
metalloproteases (Figure 1B) (32).

Because of the predicted \( \text{Zn}^{2+} \)-binding region of SELENOP1, we tested SELENOP1’s potential to bind \( \text{Zn}^{2+} \) ions by passing 
mouse serum through agarose columns bound to \( \text{Co}^{2+} \), \( \text{Ni}^{2+} \), or 
\( \text{Zn}^{2+} \), or metal-free as a negative control. Western blot of sample 
elution from each column purification shows that SELENOP1 binds to \( \text{Co}^{2+} \), \( \text{Ni}^{2+} \), and \( \text{Zn}^{2+} \), but not to the metal-free 
agarose column (Figure 1C). When serum from SELENOP1\(-/-\) mice was used, 
SELENOP1 immunoreactivity was not detected in eluent from any of the columns. This demonstrates that 
SELENOP1 is capable of binding different biometals, with a 
greater amount of binding to \( \text{Zn}^{2+} \) compared with \( \text{Co}^{2+} \) and \( \text{Ni}^{2+} \).

### Elevated Levels of Intracellular \( \text{Zn}^{2+} \) in SELENOP1\(-/-\) Hippocampus

To explore the effects of SELENOP1 on brain \( \text{Zn}^{2+} \) homeostasis, we first 
evaluated intracellular \( \text{Zn}^{2+} \) levels in SELENOP1\(-/-\) mice using histological methods. Our studies focused on the 
hippocampus as a zinc-rich region important for learning and 
memory (33). The Timm-Danscher method (34) revealed a 
pronounced increase in histologically-detectable \( \text{Zn}^{2+} \) in the 
hippocampus of SELENOP1\(-/-\) compared with SELENOP1\(+/+\) 
hippocampus (Figure 2A). The hilar and CA3 mossy fiber 
regions had the most intense \( \text{Zn}^{2+} \) labeling in both wild-type 
and SELENOP1\(-/-\) animals. However, all layers positive for 
\( \text{Zn}^{2+} \) were increased in KOs, including the stratum radiatum and 
stratum oriens of the CA1 and CA3 layers.

We further compared bioreactive \( \text{Zn}^{2+} \) levels in 
SELENOP1\(-/-\) and wildtype mouse hippocampi using TSQ 
labeling. Quantitation of TSQ fluorescence also revealed 
significantly higher levels of intracellular \( \text{Zn}^{2+} \) in the CA1 
stratum oriens, stratum radiatum, and CA3 mossy fibers 
of the SELENOP1\(-/-\) animals compared to their control 
(Figures 2B,C). We also observed a sex difference in CA3, 
with increased \( \text{Zn}^{2+} \) in mossy fibers in female compared 
to male hippocampus regardless of genotype. Unstained 
hippocampal brain sections showed no fluorescence at 
TSQ wavelengths, indicating that differences were not 
due to autofluorescence signals. Hematoxylin staining
TABLE 1 | Metal content in brain and liver of Sepp1 wildtype (Sepp1^{+/+}) and knockout (Sepp1^{−/−}) mice at the age of 3 months (mg/kg of dry tissue, \( n = 20 \) each) measured by ICP-OES.

| Tissue | Metal | Genotype | \( P \)-value* |
|--------|-------|----------|---------------|
|        |       | Sepp1^{+/+} | Sepp1^{−/−} |
| Brain  | Zn    | 12.04 ± 0.23a | 12.34 ± 0.32 | 0.45 |
|        | Cu    | 4.480 ± 0.16   | 4.383 ± 0.29  | 0.77 |
|        | Fe    | 19.99 ± 1.31   | 18.25 ± 1.04  | 0.30 |
| Liver  | Zn    | 25.85 ± 1.04   | 25.20 ± 1.48  | 0.72 |
|        | Cu    | 6.048 ± 0.42   | 6.756 ± 0.61  | 0.35 |
|        | Fe    | 88.26 ± 4.34   | 90.91 ± 7.85  | 0.77 |

*Unpaired two-tailed t-test was used to determine the probability of differences between genotypes. aMean ± SEM.

FIGURE 3 | Increased MT3 but not ZnT1 or ZnT3 protein levels in SELENOP1 KO hippocampus. Western blot showing expression levels of Zn\(^{2+}\) transporter proteins, ZnT1 and ZnT3, and Zn\(^{2+}\) storage pool, MT3. Protein densities were normalized to \( \alpha \)-tubulin and averaged to male WT values. (A) ZnT1 protein levels were unchanged. (B) ZnT3 protein was also unchanged. (C) Increased MT3 expression. Representative bands are shown above each corresponding bar graph and were found to run at the predicted sizes: ZnT1 (45kDa), ZnT3 (42kDa), and MT3 (40kDa). Statistical analysis is performed by Student’s unpaired t-test, *\( p < 0.05 \).

of sections previously used for TSQ labeling showed no morphological differences between SELENOP1 wildtype and KO hippocampi. Based on our findings, SELENOP1 may be regulating \( \text{Zn}^{2+} \) levels directly or through one or more \( \text{Zn}^{2+} \) interacting proteins.

As we observed increased levels of intracellular \( \text{Zn}^{2+} \) within the hippocampus of SELENOP1^{−/−} mice, we then investigated if the total brain \( \text{Zn}^{2+} \) levels are altered by subjecting whole brain hemispheres via ICP-OES to measure total metal content for \( \text{Zn}^{2+} \), \( \text{Cu}^{2+} \), and \( \text{Fe}^{2+} \). There was no significant increase in total brain \( \text{Zn}^{2+} \) levels in SELENOP1^{−/−} mice (Table 1). This may indicate that deletion of SELENOP1 results in changes to the distribution of \( \text{Zn}^{2+} \) within the brain rather than an increase in total brain \( \text{Zn}^{2+} \).

Expression Levels of \( \text{Zn}^{2+} \)-Interacting Proteins

We investigated whether changes in \( \text{Zn}^{2+} \) regulating proteins could explain differences in \( \text{Zn}^{2+} \). We investigated hippocampal expression of the \( \text{Zn}^{2+} \) transporters ZnT1 and the vesicle-associated ZnT3, as well as the metal storage protein MT3. Western blot indicated that ZnT1 and ZnT3 proteins were unchanged in SELENOP1^{−/−} animals (Figures 3A,B). However, MT3 protein expression was increased (Figure 3C), suggesting
that deletion of the SELENOP1 gene does not affect zinc transport through these major pathways, but rather upregulates expression of the Zn$^{2+}$ storage protein. The enhanced expression of MT3 may be a result of a feedback mechanism in response to increased Zn$^{2+}$ levels in the SELENOP1$^{-/-}$ hippocampus.

**Zn$^{2+}$ Release Is Impaired in SELENOP1$^{-/-}$ Hippocampus in Response to Neuron Depolarization**

Most histologically-detectable Zn$^{2+}$ in hippocampal neurons is vesicular (25). We investigated if the increased Zn$^{2+}$ levels visualized by TSQ or Timm’s staining in SELENOP1$^{-/-}$ mice results in increased extracellular accumulation following depolarizing stimuli designed to promote vesicular release. We imaged extracellular Zn$^{2+}$ accumulation in hippocampal slices with a selective cell-impermeant fluorescent Zn$^{2+}$ indicator, Fluozin-3, in the extracellular media. By depolarizing hippocampal cells with the addition of 35 mM KCl, we noticed an increase in fluorescence in slices from wild type mice, indicating release of Zn$^{2+}$ into the extracellular space (Figures 4A,B). In contrast, we observed a minimal increase in fluorescence in hippocampal slices from SELENOP1$^{-/-}$ mice, indicating negligible Zn$^{2+}$ release in the SELENOP1$^{-/-}$ hippocampus.

A high background fluorescence recording from Zn$^{2+}$ contaminants in the reagents used could possibly mask Zn$^{2+}$ release. To reduce background fluorescence, we also imaged slices with the Zn$^{2+}$ chelator, Ca$^{2+}$-EDTA, added to the ACSF. Ca$^{2+}$-EDTA enables removal of basal levels of extracellular Zn$^{2+}$, but its slow kinetics do not prevent the detection of synaptically-evoked Zn$^{2+}$ accumulation (35). Even in the presence of Ca$^{2+}$-EDTA, we still observed significantly larger
FluoZin-3 increases in wild-type hippocampus slices relative to SELENOP1−/− slices upon cell depolarization (Figures 4C,D). The baseline fluorescence (F₀) was not significantly different between SELENOP1+/+ and SELENOP1−/− slices in either normal ACSF for Ca²⁺-EDTA ACSF.

**Selenium Deficiency Releases Intracellular Zn²⁺**

We hypothesized that increased oxidation from Se-deficient conditions could result in reduced intracellular chelation of Zn²⁺. We tested this by measuring the fluorescence of cell-permeable FluoZin-3 in cultured SH-SY5Y cells. As shown in Figure 5A, release of intracellular zinc by DTDP, a thiol oxidizer which liberates intracellular Zn²⁺, significantly increased FluoZin-3 fluorescence. However, the intracellular Zn²⁺ chelator TPEN did not reduce FluoZin-3 fluorescence, indicating that free non-chelated Zn²⁺ levels were too low to be detectable. We also found that cells grown in 0 Se culture media had significantly increased fluorescence compared with cells grown in our baseline media with 10 nM Se (Figure 5B). Oxidation with H₂O₂ greatly increased FluoZin-3 fluorescence,
Elevated Tau Phosphorylation in SELENOP1 Knockout Hippocampus

Zn$^{2+}$ promotes tau phosphorylation leading to neurofibrillary tangle formation (18). We questioned whether tau phosphorylation could be altered in the SELENOP1$^{-/-}$ mouse. We performed western blot analysis to compare specific pTau sites to total tau protein. We found that phosphorylation at threonine 231 and at serine 396 were significantly increased in SELENOP1$^{-/-}$ mice (Figure 6A). However, phosphorylation at the serine 214 site was unchanged (Figures 6B,C). Deletion of SELENOP1$^{-/-}$ thus results in a site-specific increase in tau phosphorylation.

DISCUSSION

Our findings show that deletion of SELENOP1 increased free (chelatable) intracellular Zn$^{2+}$ levels. However, release of synaptic zinc was impaired. Although the Zn$^{2+}$ storage protein MT3 was elevated without change to the zinc transporters ZnT1 or ZnT3, we showed that selenium deficiency could induce release of Zn$^{2+}$ from stores, likely through a decrease of the selenoprotein GPX4 and a subsequent increase in lipid peroxidation. Interestingly, GPX4 helps to prevent neurodegeneration though ferroptosis (36). Lastly, we demonstrated an increase in site-specific phosphorylation of tau. These findings suggest that SELENOP1 plays a role in regulating storage of intracellular Zn$^{2+}$. This role may be important for preventing tau hyperphosphorylation in AD.

Dietary supplementation with selenium in the form of sodium selenite reduces tau phosphorylation to potentially reduce neurofibrillary tangle formation (37, 38). Selenate can act as an agonist for protein phosphatase 2A (PP2A), which targets tau phosphorylation (39). Interestingly, Zn$^{2+}$ is an inhibitor of PP2A, and may promote neurofibrillary tangle formation (18). Hyper-phosphorylation of tau leads to neurofibrillary tangle formation, and de-phosphorylation by PP2A should reduce tangle formation. However, dietary selenate supplementation also upregulates the expression of selenoproteins (40, 41). We have previously reported that a reduction in selenoprotein S can promote tau phosphorylation (42). In a type 2 early clinical trial for selenate supplementation in Alzheimer’s disease, selenate could increase brain selenium in some patients, and the increase in brain selenium correlated with lack of decline in performance on the Mini-Mental Status Examination (MMSE) (43). Thus, brain selenoproteins, including SELENOP1, may be important for preventing Alzheimer’s pathology.

Zn$^{2+}$ metabolism is altered in AD, resulting in abnormally enriched Zn$^{2+}$ environments within the AD brain (44). Zinc-binding sites on the Aβ peptide result in Zn$^{2+}$ mediated aggregation of Aβ and amyloid plaque formation. Furthermore, both the neuroprotective role of SELENOP1 against Aβ toxicity and the role of Zn$^{2+}$ in protecting the cell against oxidative damage, could be working together to reduce the levels of Aβ stress observed in AD pathology, however more studies need to be done to further elucidate their contribution to alleviating oxidative stress.

We did not observe an increase in total brain Zn$^{2+}$ levels with ICP-OES, suggesting only changes in local Zn$^{2+}$ distribution.
The absence of SELENOP1 could result in increased oxidative stress in the brain and lead to Zn$^{2+}$ release from MT3 (45), possibly inducing an upregulation of the Zn$^{2+}$ storage protein. In the absence of SELENOP1, we observed a seemingly paradoxical impairment of Zn$^{2+}$ release despite an overall increase in intracellular chelatable Zn$^{2+}$. This was surprising, since most chelatable Zn$^{2+}$ is thought to be localized to synaptic vesicles (46). Overexpression of MT3 in SELENOP1$^{-/-}$ mice may affect the subcellular distribution of Zn$^{2+}$ by limiting the amount of free Zn$^{2+}$ available for loading into the synaptic vesicles. The protein Reelin can increase release of a subset of synaptic vesicles (47). This increase is dependent on Reelin binding to ApoER2. SELENOP1 is another ligand for ApoER2 (48, 49), and thus may also modulate vesicle release, possibly including zincergic vesicles. A decrease in release of Zn$^{2+}$ vesicles could result in a “back-up” of these vesicles, which could contribute to the observed increase in chelatable Zn$^{2+}$. The APOE ε4 allele of the major ligand ApoE for ApoER2, increases risk of Alzheimer’s disease (50). Interestingly, the APOE ε4 allele is also associated with decreased selenium in the brain (51), again suggesting a possible role for selenium and selenoproteins in preventing Alzheimer’s disease.

We found an interesting sex difference in Zn$^{2+}$ levels in the CA3 region of the hippocampus (Figure 2). Female mice generally had higher Zn$^{2+}$ levels but with some variability. Previous studies have shown higher Zn$^{2+}$ levels in female wild-type mice and AD mouse models (52, 53), although these studies did not agree on the age of sex differences. The differences suggest Zn$^{2+}$ as a possible explanation for the increased risk of AD in women (54). Researchers recently discovered that estrogen increases hippocampal Zn$^{2+}$, which was cycle-dependent in female mice (55, 55), which may explain the increased Zn$^{2+}$ in other studies as well as the variability of the current results.

SELENOP1 contains a putative metal-binding domain that can potentially bind Zn$^{2+}$ with a high affinity. Our results demonstrate that SELENOP1 is capable of binding Zn$^{2+}$ as well as Co$^{2+}$ and Ni$^{2+}$. However, our finding that selenium deficiency and inhibition of GPX4 suggest that the absence of selenium and loss of antioxidant selenoprotein function in the SELENOP1 KO mice is responsible for the increased Zn$^{2+}$ levels. The reason for the presence of the functional metal-binding domain of SELENOP1 remains unknown. It is possible that Zn$^{2+}$ binding could alter the affinity of SELENOP1 for the ApoER2 receptor, allowing for regulation of Se by excess Zn$^{2+}$. Additionally, the homology of the Zn$^{2+}$ binding domain with metalloproteases such as ADAM10 and nephrilysin (Figure 1) opens the possibility of an enzymatic role in proteolysis. ADAM10 has a protective role in Alzheimer’s disease as a putative α-secretase that promotes non-amyloidogenic cleavage of amyloid precursor protein, preventing amyloid beta formation (56).

The data presented here indicate that SELENOP1 may play a crucial role in the maintenance of brain Zn$^{2+}$. The SELENOP1 gene can affect Zn$^{2+}$ metabolism and synaptic release from neuronal synapses. Zn$^{2+}$ is increased in Alzheimer’s disease and interacts with amyloid beta (17), and can also promote tau phosphorylation (18). Thus, Zn$^{2+}$-binding properties of SELENOP1 could contribute to the association of SELENOP1 with amyloid beta plaques in Alzheimer’s disease (8). The SELENOP1-Zn$^{2+}$ interaction has potentially important implications in neuronal function and synaptic physiology.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

**ETHICS STATEMENT**

The animal study was reviewed and approved by University of Hawaii IACUC.

**AUTHOR CONTRIBUTIONS**

FB, CS, DT, and AK designed the research. DT, AH, JP, and RR performed the research. FB analyzed the data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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