DISTINCT ROLES OF SELENOPROTEINS IN FACILITATING SYNAPTIC PLASTICITY FOR LEARNING AND MEMORY

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

CELL AND MOLECULAR BIOLOGY (NEUROSCIENCES)

JULY 2017

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Keywords: Selenoprotein, Oxidative Stress, Neurodegeneration, Synaptic Plasticity

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ApoER2	apolipoprotein E Receptor 2 (aka LRPR8)
DIOs	deiodinases
Dio1	iodothyronine deiodinase 1
DAPI	4',6-diamidino-2-phenylindole
DTT	dithiothreitol
ER	endoplasmic reticulum
GPx	glutathione peroxidase
GSH	glutathione
H_2O_2	hydrogen peroxide
NGS,	normal goat serum
PFA	paraformaldehyde
ROS	reactive oxygen species
SBP2	selenocysteine insertion sequence binding protein 2
Se	selenium
Sec	selenocysteine
SECIS	selenocysteine insertion sequence
SecS	selenocysteine tRNA Synthase
Sel I	selenoprotein I
SelH	selenoprotein H
SelK	selenoprotein K
SelM	selenoprotein M
SelN	selenoprotein N
SelO	selenoprotein O

SelR	selenoprotein R
SelS	selenoprotein S
SelT	selenoprotein T
SelV	selenoprotein V
SelW	selenoprotein W
SeMet,	selenomethionine;
Sepp1	selenoprotein P
SPS2	selenophosphate synthetase 2
TBS	tris-buffered saline
$tRNA^{[Ser]Sec}$	selenocysteine tRNA
TRxR	thioredoxin reductase
TRx	thioredoxin
U	Sec

ABSTRACT

Selenium (Se) is a micronutrient essential for life and important for proper neurological and immune function, reproductive viability, and cardiovascular health. In the body, it acts through incorporation into selenoproteins, which can function as antioxidants that protect cells from oxidative damage and maintain many other cellular functions. This dissertation focused on the roles of Selenoprotein P and M (Sepp1 and SelM) in learning and memory.

Sepp1 is primarily thought to transport Se to the tissues, including the brain, for synthesis of other selenoproteins. Sepp1 has been shown to colocalize with amyloid beta plaques in postmortem brains from patients diagnosed with Alzheimer's disease (AD) (1). Sepp1$^{-/-}$ mice on selenium deficient diets have severe neurological impairments with major motor function deficits, and impaired hippocampal dependent synaptic function and memory, similar to deficits seen in AD. We hypothesize that Sepp1 has a localized function in the brain, independent of its function in transporting selenium to the brain and other body tissues.

SelM has antioxidant properties and is highly expressed in the brain (2). The hippocampus, one of the areas in which SelM is expressed, is involved in learning and memory acquisition. A study using an AD mouse model having a mutant form of presenilin-2 resulted in suppression of SelM expression (3). We report here that SelM$^{-/-}$ male, but not female mice, lack hippocampal long-term potentiation (LTP), which is a cellular model for learning and memory. These results suggest that SelM has an integral sex-specific role in synaptic plasticity, learning and memory.

To determine the direct role for Sepp1 in the brain, we developed a novel mouse model that has restored SEPP1 expression in forebrain neurons of Sepp1$^{-/-}$ mice. The successful restoration of LTP in Sepp1$^{-/-}$ mice with locally restricted SEPP1 gene rescue to forebrain neurons highlights the critical role Sepp1 plays in synaptic plasticity as well as enabling synthesis of SelM and other selenoproteins that are required for learning and memory. We elucidate the important neuroprotective properties of these selenoproteins in memory and learning, serving as a foundation for further studies to understand their roles in Alzheimer's disease and other neurodegenerative disorders.

CHAPTER I

INTRODUCTION

Selenium (Se) is a nutritional trace mineral essential for human health that is acquired primarily through dietary intake. The recommended daily allowance (RDA) for adults is 55µg/day, with 400 µg/day as the tolerable maximum before the onset of adverse effects (4).The primary source of Se is found initially in soil which is converted into nutrients for crops and livestock. Although soil and food in most regions of the United States have adequate selenium, some soils in other parts of the world, such as China and New Zealand, are selenium deficient.

Se is incorporated into selenoproteins as the 21st amino acid, selenocysteine. Many of the twenty-five known selenoproteins in humans, of which 24 are also found in rodents, have antioxidant properties that protect cells from oxidative stress (5–7). Se deficiency and/or mutations and polymorphisms in selenoprotein genes and cofactors (8) have been implicated in many diseases and conditions including neurodegeneration (1), infertility (9,10), cardiovascular disease, immune disorders, and cancer (11–13) in humans; (14). Low plasma selenium levels have been associated with intestinal malabsorption syndrome, cystic fibrosis, rheumatoid arthritis, neoplasia, and other clinical disorders in human beings (15). In certain Se-deficient areas of China, in which the population received less than 10 µg Se per day, led to development of Keshan disease, a prevalent but preventable cardiomyopathy that can be treated with Se supplementation (16,17). While Se is a vital micronutrient, excessive Se intake can lead to toxicity, a condition known as selenosis. Excessive chronic consumption of more than 910 µg Se per day can lead to clinical selenosis in which symptoms such as hair loss, brittle hair, gastrointestinal disturbances, and a garlic odor from the breath and skin are exhibited and in extreme cases can lead to death (18,19).

Despite much progress in studying members of this unique family of proteins, the properties and functions of many of the selenoproteins are still not clearly understood. Vertebrate selenoproteins have been highly conserved through evolution. Coupled with the fact that Se is required for life and implicated in a host of diseases, further

elucidating the diverse functions and mechanisms of the family of selenoproteins may make critical contributions to treating many diseases and health conditions. This chapter provides an overview of the current understanding of Se and members of the selenoprotein family, including two that are central to this study, selenoprotein P (Sepp1) and selenoprotein M (SelM).

Brief History of Selenium

The discovery of selenium dates back to 1817 when Jöns Jacob Berzelius, a Swedish chemist, stumbled upon what he thought was tellurium, as he was analyzing contaminants in sulfuric acid produced in his factory(20). Berzelius' discovery of Se, which he named after the Greek moon goddess, Selene, established him as one of the world's leading chemists in the 19th century.

Se can be found directly below sulfur and above tellurium in the periodical table and shares similar chemical properties with sulfur (21). Se is a fairly reactive element and combines directly and hydrochemically with both metals and non-metals (21). It combines easily with hydrogen, fluorine, chlorine, and bromine and reacts with nitric and sulfuric acids. It forms compounds called selenides when combined with certain metals. Se has chemical characteristics similar to sulfur and can quickly become toxic at high doses; five times more poisonous than arsenic (21). Not only does Se dosage and accumulation have a narrow range between safety and danger, but the range of optimal beneficial action is very narrow (21). For this reason, Se was more widely known as a toxin to livestock and people in industrial jobs with reports of teratogenic effects and carcinogenicity. It was not until 1954 that the biological functions of Se in microorganisms were first reported (22).

Finally in 1957, 140 years after the discovery of selenium, Schwartz and Foltz published a seminal manuscript describing this essential trace element as a critical protective dietary component against necrotic liver degeneration in rodents. Since then, extensive research has further affirmed that selenium is in fact required for cellular function in most terrestrial and some aquatic animal life. In the 1970's, biochemist Thressa Stadtman (23) made the critical discovery of selenocysteine (Sec), Eventually, this lead recognition of Se as the 21st amino acid. Se has unique properties in that it is

encoded by the UGA codon, which is normally a stop codon. The mechanism in which this recoding event occurs was not uncovered until the 1980's. These early discoveries in the Se field have paved the way to a vast expansion in Se research that encompasses multiple areas of human health and disease, veterinary medicine, and plant sciences.

Research on selenium and its biological effects on human health has also grown immensely. Many studies and large clinical trials have been conducted to explore whether dietary selenium can be used as a therapeutic tool for a wide range of medical conditions including cancer, infertility, and immune disorders. A 4.5-year study, using a supplementation of 200 µg/day Se as selenized yeast, showed that two-thirds of participants had lower risk of developing prostate cancer, but it was the men with initially low Se levels who benefitted the most (24). Another study revealed that low plasma selenium was associated with a 4 to 5-fold increased risk of prostate cancer (25). This lead to the Selenium and Vitamin E Cancer Prevention Trial (SELECT) trial, one of the largest cancer prevention studies conducted, which reported that Se or vitamin E, alone or in combination, at the doses and formulations used (Se=200 µg/day from L-selenomethionine), vitamin E= 400 IU/d) did not prevent nor increase the risk of prostate cancer in this population of relatively healthy men (26,27).

In other studies that compared the level of zinc, selenium, glutathione peroxidase activity and antioxidant status in men with severe prostate inflammation, severe leukocytospermia, non-inflammatory oligozoospermia, and male partners of infertile couples, it was found that reduced levels of antioxidant activity and selenium levels in their seminal plasma regardless of inflammation status (28).

In young adults, it was also found that an optimal range of serum selenium between ~82 and 85 µg/L was associated with reduced risk of depressive symptomatology (29) and Se-enriched diets not only prevent methylmercury (MeHg) toxicity, but can also rapidly reverse some of its most severe symptoms (30,31). These are just some examples of the numerous studies which demonstrate that a greater understanding of the biological functions of selenium and selenoproteins is warranted in order to harness this knowledge towards improving human health and the critical role it plays in all living organisms.

The Selenoprotein Family

Selenium (Se) is a requisite micronutrient present in all major life forms, including eukaryotes, bacteria and archaea (32). This trace element is incorporated into selenoproteins as the 21st amino acid, selenocysteine (Sec) (33). Sec has a structure that is nearly identical with that of cysteine, with the exception of a single atom, selenium, in place of sulfur, giving Sec a lower pKa (5.2 versus 8.3) and higher reactivity (8). Selenoproteins can be up to two orders of magnitude more effective in catalysis than their cysteine homologs (34,35). This is most likely one of the major reasons that nature has invested in evolving Se-dependent pathways and the specialized machinery used for Se insertion into protein.

Selenoproteins are present in most life forms. Selenoproteins often function as antioxidants in higher organisms, but are rare or absent in plants and fungi, with the exception of the green alga Chlamydomonas (32,36). Many components of the selenocysteine biosynthesis pathway have been conserved in archaea and eukaryotes that have selenoproteins (37). While there are more than 25 known selenoprotein genes found in vertebrate genomes, 25 in human beings and 24 in mice (32,38,39), there are only a few present in invertebrate genomes, three in *Drosophilia melanogaster* and one in Caenorhabditis *elegans* (32,40,41).

Se exists in inorganic (selenate and selenite) and organic forms (selenomethionine and selenocysteine) (21,42). The bioavailable source of Se comes in many forms, but for most organisms, it occurs in the organic form of selenomethionine, and in the inorganic sodium selenate (i.e. supplements and animal food) (43). Both forms are sufficient sources of dietary Se, however metabolic studies report selenomethionine is more effectively absorbed and retained than selenite (44,45). Selenomethionine, a naturally occurring amino acid, is incorporated randomly in place of methionine. This is the most common source of selenium for livestock consuming natural feeds (46). L-selenomethionine is the primary form of selenium found in common foods such as Brazil nuts, cereal grain, legumes, and soybeans (47). Selenomethionine is absorbed in the small intestine via the sodium-dependent amino

acid transport system and the remaining that is not immediately metabolized is incorporated into organs with high rates of protein synthesis into the skeletal muscles, erythrocytes, pancreas, liver, kidney, stomach, the gastrointestinal mucosa, as well as a significant amount in the brain (44,48,49). Selenite and selenates are produced by soil microorganisms from less soluble forms of selenium that accumulate, and are converted to organic forms, mostly selenocysteine and selenomethionine and their methylated derivatives (42,50). Selenoprotein biosynthesis can be mediated by selenite and selenates but only selenomethionine is incorporated into proteins to be stored in the body and utilized through metabolic processes (44). Selenite, unlike selenomethionine, exerts its antioxidative effect indirectly and requires de novo synthesis (51). The beneficial range of selenium is quite narrow where either deficiency or excess can have adverse health effects. Selenite toxicity has extensively been shown to act through oxidative stress and generation of reactive oxygen species (ROS) (52). The mechanism for selenium toxicity through selenomethionine is not as well understood. A study in *Saccharomyces cerevisiae* suggest that selenomethionine toxicity involves production of superoxide radicals mediated by the trans-sulfuration pathway amino acids selenohomocysteine and/or selenocysteine, which are more reactive and are capable of generating superoxide radicals upon oxidation (52).

Selenoprotein Function and Metabolism. Selenium containing proteins were originally detected by radioactive Se^{75} labeling (53,54) and they exhibit diverse tissue allocation. Subcellular localization also varies greatly, with some selenoproteins solely expressed in certain organelles or as transmembrane proteins, while others are secreted to extracellular spaces or plasma (8,55). Most selenoproteins can be categorized into two main groups according to the location of the Sec residue (Table 1) (34). One group of selenoproteins contain Sec very close to the C-terminus of the protein. Mammalian selenoproteins K, S, O, I, R (MsrB) and thioredoxin reductases (TRxRs) are examples of such proteins (34). Other proteins, such as mammalian selenoproteins H, M, T, V, W, Sep15, selenophosphate synthetase 2 (SPS2), thyroid hormone deiodinases (DIOs) and glutathione peroxidases (GPxs), have Sec in the N-terminal segments of proteins, and often as part of the CxxU motif that corresponds to

the CxxC motif (two cysteines separated by two residues) in the active site of thioredoxin (34). Several selenoproteins have been characterized as antioxidant enzymes that alleviate damage caused by reactive oxygen species (ROS) (5–7), and may have potential roles as modulators of redox-regulated signal transduction (5).

Three classes of selenoproteins, the GPxs, TRxRs and DIOs were among the first eukaryotic selenoproteins discovered and are the most extensively studied (8). The GPxs, the largest selenoprotein family in vertebrates, are integral to antioxidant glutathione pathways, providing protection from ROS, and are critical for antioxidant defense in humans (56). Five of the eight GPxs in humans (four in mice) are selenoenzymes (57,58). GPxs are hydroperoxidases that reduce hydrogen peroxide and alkyl hydroperoxides, using glutathione as a cofactor. The main reaction that glutathione peroxidase catalyzes is 2GSH + H2O2 → GS–SG + 2H2O (Fig. 3) (42).

Members of the GPx family of enzymes vary in subcellular localization, substrate specificity, and some have multiple transcript variants and protein isoforms. GPx1, one of the first selenoenzymes discovered, is primarily found in cytoplasm of mammals and reduces hydrogen peroxide and organic hydroperoxides (59,60).GPx2 and GPx3 are extracellular enzymes with the former found in the intestines and the latter mainly in plasma (61). GPx4 differs from the other members in that it is a phospholipid hydroperoxidase that protects cells membranes from lipid peroxidation and is expressed in nearly all mammalian cells. GPx4 catalyzes the reduction of hydroperoxides and lipid peroxides producing the oxidized form of glutathione (GSH), glutathione disulfide (GSSG), which is then recycled by glutathione reductase and NADPH/H^+. GPX4 contains a selenocysteine as well as several cysteines with the ability to reduce protein thiols (62)

There are three known isoforms of GPx4 that differ in subcellular location; cytosolic, mitochondrial (mGPx4), and nuclear (nGPx4). The last two have been implicated in recent studies to be essential for spermatogenesis and male fertility(63,64). The nuclear isoform of GPx4 is expressed in male germ cells. In knockout studies of GPx1, GPx2, GPx3 and GPx4 null mice, GPx4 is the only one that is embryonic lethal. Ran et al., whom showed that GPx4 deletion caused lethality, also generated an overexpressing GPx4 transgenic mouse model showing that other major

antioxidant enzymes such as Cu/Zn superoxide dismutase, manganese superoxide dismutase, particularly GPx1, and catalase, levels remained unaffected in their model and therefore concluded that GPx4 is uniquely involved in the ability to remove lipid hydroperoxides (65). Taken together, evidence from many studies indicates that of all the GPx's, GPx4 is critical for embryonic development, prevents oxidative stress induced apoptosis in several cell types thereby increasing cell survival (66–68).

Se and at least eight selenoproteins (deiodinase 1 (Dio1), Dio2, Dio3, thioredoxin reductase 2 (Txnrd2), selenoprotein I (SelI), selenoprotein U (SelU), glutathione peroxidase 1 (Gpx1), and Gpx2) has been shown to affect thyroid hormone metabolism in mammals (69). Thioredoxins (TRx) are involved in selenium metabolism, reducing selenium compounds and thereby providing selenide to synthesis of all selenoproteins (70). The TRxRs use NADPH for reduction of TRx in cellular redox pathways and have been found to be involved in cell proliferation, and clinical conditions including cancer, cardiomyopathy, and has been suggested to have a therapeutic role in for HIV/AIDS patients (71,71,72).

The iodothyronine deiodinases are involved in homeostatic function of thyroid hormone (73), thermogenesis, and play key roles in development and growth of the cardiovascular and muscle-skeleton systems, and cognitive function (74,75). Deiodinase types 1, 2 and 3 are encoded by DIO1, DIO2, DIO3, and belong to the iodothyronine deiodinase family. These enzymes catalyze release of iodine directly from the iodothyronine hormones (73). The identification of Sec in the active site of rat Deiodinase type 1 (D1) led the way to elucidate the key requirements for and mechanism of Sec incorporation in eukaryotes as well as the cDNAs that encode Deiodinases types 2 and 3 (74,76).These selenocysteine-dependent membrane proteins contribute to activation and inactivation of the initially released hormone precursor thyroxine (T_4) into triiodothyronine (T_3) or reverse triiodothyronine (rT_3) in target cells (77). The enzymes catalyze a reductive elimination of iodine in which the different isoforms attack different iodine positions, oxidizing themselves, thus followed by a reductive recycling of the enzyme (77).

Selenoprotein W (SelW) is highly expressed in skeletal muscle and found to be involved in white muscle disease in livestock (78). It is similar to the GPx family in that it

shares the redox motif and binds glutathione (79). Selenoprotein H (SelH) is a nuclear-localized DNA-binding protein that may act as a transcription factor that is involved in cellular oxidative stress response (8,80). Selenoprotein I (SelI) was found to be the mammalian form of the phospholipid-synthesizing enzyme ethanolamine phosphotransferase (8,81). Selenoprotein R (SelR, also previously known as selenoprotein X (SelX) and also referred to as MsrB1) is a member of the methionine sulfoxide reductase family, important for reduction of sulfoxymethyl groups (8). Selenoproteins O and V (SelO and SelV, respectively) are two of the least known in this family. SelO is widely distributed, whereas SelV expression is limited to testes (32). Selenoprotein N (SelN) is localized to the endoplasmic reticulum (ER) membrane and may be necessary for proper muscle development (82). Selenoprotein S (SelS) is also ER-localized and is important for removal of misfolded proteins from the ER membrane (83).

Selenoproteins K, M and T (SelK, SelM and SelT, respectively) and the 15 kDa selenoprotein 15 (Sep15) are small ER proteins whose functions are under investigation (55). SelT and Sep15 have both been reported to have antioxidant properties as well (84–86). Recent studies show SelT is expressed in pancreatic β- and δ-cells and is regulated by pituitary adenylate cyclase-activating polypeptide (PACAP) and is involved in the control of glucose homeostasis. The role of SelK in vertebrate function was recently found to be involved in atherosclerosis and palmitoylation (87). SelK is also involved in calcium Ca^{2+} flux in immune cells, the ER-associated protein degradation pathway, and was most recently reported to have an intermolecular diselenide bond with unusually high redox potential (88,89).

Selenoprotein P (Sepp1 or SelP), one of the more well studied selenoproteins, is extracellularly released protein that transports Se from liver to other tissues and hence enables selenoprotein biosynthesis(1,3,90). Sepp1 has been implicated in having a protective function in conditions including neurodegeneration in Alzheimer's disease (1,3,91). The critical role of Sepp1 in the nervous system will be discussed in further detail in this dissertation. This unique selenoprotein has also been suggested to play a critical role during development and normal male fertility (10). Numerous studies have shown that selenium plays an important role in reproductive health (9,92–94). Selenium

deficiency has been reported to be involved in several reproductive and obstetric complications including male and female infertility, miscarriage, preeclampsia, fetal growth restriction, preterm labor, and gestational diabetes (95).

SelM was first discovered in 2002, when it was reported that human SelM is a 3 kb gene containing 5 exons on chromosome 22 (96). The 3"UTR of the genes encoding rat, mouse, and human SelM lacks a canonical SECIS element but instead contains a conserved mRNA structure, in which cytidines take the place of adenosines but was previously considered invariant (96). This new form of the SECIS element in SelM was found to be able to still facilitate incorporation of the Sec (96). The 15 kDa SelM protein is localized to the ER with a thioredoxin-like domain (CxxU), suggesting an oxidoreductase function, catalyzing the reduction or rearrangement of disulfide bonds in the ER-localized or secretory proteins (97). The N-terminal peptide was confirmed to be necessary for protein translocation (96). Sep15 and SelM have a sequence homology of 31% (97). However, it has a COOH-terminal extension with an ER retention signal that is highly flexible and therefore may participate in substrate binding or interaction with other protein factors (97,98). SelM is expressed in the heart, lung, kidney, stomach, intestine, skin, uterus, and placenta, but is most abundant in the brain (96). A recent study examined the structure-function relationship of SelM in hepatoma cell lines and primary hepatocytes in which SelM expression was upregulated in both mRNA and protein analysis (99). The results of the study suggests a role for SelM and its potential use as biomarker for hepatocellular carcinoma (99). However, most published studies on SelM have focused on its role in the brain. SelM has been shown to have neuroprotective functions and to be involved in regulating energy metabolism (2,100). Many selenoproteins have largely unknown functions, with some less well characterized than others. The known properties and functions of all selenoproteins are briefly summarized in Table 1. In this study we will focus on selenoprotein P and seloprotein M, briefly discussed here and in further detail throughout this body of work.

Selenium Distribution and Synthesis of Selenoproteins

Hierarchy of Se Distribution

Selenium cycling through Sepp1 occurs at a high rate as indicated by the 3-4hr half-life of this protein in plasma (101). Roughly 25% of whole-body selenium passes through rat plasma daily (102). Plasma Sepp1 most likely supports homeostatic expression of GPxs, TRxRs and other selenoenzymes (8) through its role in supplying selenium to cells throughout the body (103). Deletion of Sepp1 differentially affects selenium supply to various tissues (104) and, therefore, affects selenoprotein levels, selenoprotein-synthesis factors, and also the turnover of selenoprotein mRNAs via the nonsense-mediated decay pathway (104). Se remains stable in testes and brain relative to other tissues, even in low Se conditions (101), in which Sepp1 may potentially be broken down to recycle Se or remain circulating in blood while gradually being taken up in brain and testes. Under low dietary selenium conditions, selenocysteine incorporation is inefficient, resulting in some selenoprotein mRNAs being degraded via nonsense-mediated decay (105). Selenium deficiency down-regulates Gpx1 mRNA to 15% of the selenium-replete value, while reducing Sepp1 mRNA, the most abundant hepatic selenoprotein mRNA, only to 61% (106). This strongly suggests that Sepp1 synthesis is favored in the liver over Gpx1 synthesis when selenium supply is limited, directing hepatocyte selenium to peripheral tissues in selenium deficiency (106).

Nonsense-mediated decay is a pathway that targets for degradation mRNAs containing premature termination codons (107). The presence of both a UGA codon and an RNA element downstream of the UGA were shown to be necessary for selenium-dependent regulation of mRNA turnover (105). Degradation of selenoprotein mRNAs under conditions of low Se is not uniform, with some transcripts being more sensitive to nonsense-mediated decay than others (108,109). Several factors may contribute to the sensitivity of selenoprotein mRNAs to nonsense-mediated decay (110) at different steps of the translation process (111). The energetically demanding, yet uniquely conserved process of incorporating the 21st amino acid, Sec and its translational mechanism that is utilized by a diverse family of proteins underscores how critical selenoproteins are for cellular function in many organisms throughout evolution(112).

Selenoprotein Synthesis

Selenocysteine (Sec) biosynthesis is unique in that it takes place on its tRNA, which recognizes the UGA codon, typically a stop codon (113). Thus, recoding of the UGA codon, located in the coding region of selenoprotein mRNAs, from a stop codon to a selenocysteine-insertion codon is required. During the unique translational mechanism of selenoprotein synthesis, *cis*- and *trans*-acting factors work synchronously to redirect translational machinery to insert selenocysteine at UGA codons instead of terminating polypeptide synthesis. These factors include an element in the 3′-UTR (untranslated region) of eukaryotic selenoprotein mRNAs, termed the Sec insertion sequence (SECIS), which was discovered following the cloning of another selenoprotein, iodothyronine deiodinase 1 (Dio1) (8,114). The selenocysteine insertion sequence (SECIS), originally studied in bacteria, is a RNA element with a unique stem-loop structure that directs the cell to translate the UGA codon as Sec instead of a stop codon. The SECIS elements in bacteria, archae, and eukaryotes are distinct in structure, position and mechanisms of action (114,115). For instance, yeast and higher plants do not possess selenoproteins, as the Sec insertion machinery was lost during evolution (34). In these organisms, cysteine-containing homologs of some selenoproteins are utilized instead (34).

All eukaryotic selenoproteins require a form of the SECIS element for recoding UGA to the Sec codon (116). The translational machinery within the cell typically identifies the UGA codon as a termination signal, thereby releasing the nascent polypeptide from the ribosome (115). The biosynthesis of selenocysteine on $tRNA^{[Ser]Sec}$ is catalyzed by selenocysteine synthase (117,118) and the $tRNA^{[Ser]Sec}$-modifying enzyme phosphoseryl-$tRNA^{[Ser]Sec}$ kinase (111). The translation process also requires a SECIS-RNA binding protein (SBP2) (119,107) which recruits a specialized elongation factor (120,121), that delivers selenocysteyl-$tRNA^{[Ser]Sec}$ to the A-site of the ribosome. Another Sec-$tRNA^{[Ser]Sec}$ binding protein, SecP43, is required for methylation of the 2'-hydroxyl-ribosyl moiety in the wobble position of the selenocysteyl-$tRNA^{[Ser]Sec}$. The assembly of this selenosome protein complex may regulate the shuttling of the selenocysteine synthase-selenocysteyl-$tRNA^{[Ser]Sec}$ complex between the nucleus and

cytoplasm for early recruitment of the complexes on pre-mRNAs to circumvent NMD (Fig. 1) (122,123).

Selenoproteins in Brain Function

Selenium and many selenoproteins have protective functions in neuropathology. Selenium deficiency causes epileptic seizures in both humans and mice and supplementation has shown to reduce seizures (113,124). Selenium protects neurons even under glutathione depletion (125). Several important selenoproteins with antioxidant functions are expressed in the brain, and may mitigate neurodegeneration (126). One such selenoprotein is GPx4 in which neuronal specific deletion causes severe neurodegeneration (57) and furthermore genetic deletion of GPx4 is embryonically lethal (127,128). GPx4 is not only involved in cerebral embryogenesis and hindbrain development (129) but is also expressed in neurons of cerebral cortex, hippocampus, and cerebellum but absent in glial cells of the adult brain (130,131), protecting them from oxidative injury (131,132). Interestingly, following brain injury specifically in the hippocampus, frontal and entorhinal cortex, cytosolic GPx4 (cGPx4) is highly upregulated in reactive astrocytes in the immediate area of induced brain lesions and the surrounding zone of deafferentiation where neurodegeneration was occurring (130,131). Upregulation of GPx4 expression was shown to be a beneficial effect, rescuing the cells from apoptosis, preventing further cell damage at and around sites of induced brain injury, determined by *in vitro* siRNA and apoptotic induction experiments (130,131).

GPx1 has been shown to have a protective role in Parkinson's Disease (PD), in which severe loss of dopamine-releasing neurons in the substantia nigra occur (8,133). In PD mouse models, deletion of GPx1 significantly increased dopamine loss and PD pathology, while overexpression had a protective effect (8,134,135). Neuronal system-specific knockout of TRxR1 causes severe neurological symptoms, such as ataxia and tremor due to cerebral hypoplasia (57). Glutathione peroxidase 1 knockout ($GPx1^{-/-}$) mice are viable and appear healthy when on a Se adequate diet, but exhibit increased susceptibility to oxidative stress-inducing agents paraquat and H_20_2, wherein 30% of

neurons were killed, compared to wildtype controls on the same diet that were not affected (136).

Extensive research has shown that many selenoproteins are involved in neurological development and function. In this investigation, we will primarily focus on selenoproteins P and M in neurological function, their significant roles in learning and memory, and how these new findings contribute to furthering our understanding of neurodegenerative conditions.

Selenoprotein P

Selenoprotein P (Sepp1) was first identified through biochemical studies in 1982 (137). Sepp1 is an unusual selenoprotein, in that it contains multiple Sec residues; ten Sec residues in humans and rodents, 16–18 in amphibians and fish, and 28 in sea urchins (8), while most selenoproteins typically have only one Sec residue per polypeptide chain. The C-terminal domain of Sepp1 contains nine Sec residues which are thought to be critical for the maintenance of Se levels in the brain and testes. The N-terminal domain has only one Sec residue and potentially has antioxidant functions(101,138,139). Transgenic mice lacking the Sec-rich C-terminus exhibit severe Se deficiency in brain in the absence of dietary Se supplementation, and have greater susceptibility to infections and morbidity (103,113).

Sepp1 is primarily synthesized and secreted from hepatic cells to deliver selenium to other tissues and organs in the body (Fig. 2) (102). Sepp1 plays a central role in selenium homeostasis particularly in the brain and testes. Sepp1 has been found to be highly expressed in the liver, testes - specifically the Leydig cells, kidneys, and in lesser amounts in the gut and hematopoietic cells (140,141). Sepp1 is also synthesized in smaller amounts in the brain, where it has been found in glial cells and choroid plexus cells (142). Under selenium-deficient conditions, selenium is better retained in the testes and brain relative to the other organs suggesting that selenium is critical for maintenance of these tissues (143).

Sepp1 is a ligand for the low-density lipoprotein (LRP)-related receptors apolipoprotein E receptor 2 (ApoER2 or LRP8) and megalin (LRP2), which may have roles in AD. ApoER2 facilitates the uptake of Sepp1 into the testis and allows retention

in the brain. In an elegant study by the Burk lab investigating the mechanism of selenium transport from systemic circulation to the brain, it was found that Sepp1 binds to ApoER2 by day 18 in fetal brain blood brain barrier (BBB) capillaries and choroid plexus (CP) (142) endothelial cells, suggesting that Sepp1 is in vesicles as well (142). Sepp1 and apoER2 were present in the same cellular compartment, while deletion of apoER2 abolished Sepp1 association with the choroid plexus, suggesting ApoER2-mediated endocytosis of Sepp1 (142).

Megalin (also known as LRP2), a member of the low-density lipoprotein receptor family (LDLR), is expressed in many tissues including the brain, in ependymal and choroid plexus cells (144–147). Megalin facilitates endocytosis of filtered Sepp1 isoforms via the renal proximal convoluted tubule (PCT) cells (142,148). Deletion of Megalin results in severe malformations in the brain and other tissues, and most mice die within minutes after birth (149). Megalin null mice exhibit impaired proliferation of neuroepithelium that produced a holoprosencephalic syndrome, characterized by lack of olfactory bulbs, forebrain fusion, and a common ventricular system (150). Genetic deletion of megalin did not eliminate the presence of Sepp1 in either capillary or choroid plexus cells in day18 fetal brain and did not affect brain selenium concentration in adult mice fed a selenium-adequate diet (142). This particular study by Burk et al. has been the only study on the interaction of megalin and Sepp1 in the brain. Although it did not provide direct evidence for a megalin-Sepp1 interaction in the brain, neither did it rule one out (142).

When selenium is limited, Sepp1 synthesis has priority over glutathione peroxidase synthesis (53, 57,58). Presumably, astrocytes secrete Sepp1, which is subsequently taken up by neurons via the ApoER2(153). Knock-out of Sepp1 or ApoER2 as well as neuron-specific ablation of selenoprotein biosynthesis results in neurological dysfunction in mice. Astrocytes, generally less vulnerable to oxidative stress than neurons, are capable of up-regulating the expression of antioxidant selenoproteins upon brain injury (154).

Sepp1 also has important functions in normal male reproduction as well as during embryonic development. Sepp1 is suggested to function in oxidant protection (10,103,155) and has been shown to promote the survival of cells in culture (7,10).

Maintaining Sepp1 homeostasis is critical for overall Se balance. Impaired GPx4 biosynthesis, due to selenium deficiency or to genetic defects in GPx4 or Sepp1, has been implicated in conditions such as infertility (93). The relationships between Sepp1, GPx4, and other selenoproteins in human diseases are important and merit further investigation.

Selenoprotein P is required for normal brain function. Evidence shows that Sepp1 gene deletion or Se deficiency lowered Se by similar amounts in cortex, midbrain, brainstem and cerebellum. However, Se in hippocampus was lowered by genetic deletion of Sepp1, but not by Se deficiency, suggesting that Sepp1 is more important for maintaining Se in hippocampus than in other brain regions (156). Mutant mice lacking the Sec-rich C-terminus exhibit severe deficiencies in brain Se if not supplemented in the diet and have greater susceptibility to infections and morbidity (113,157). Deletion of Sepp1 led to reduced Se content in plasma, kidney, testis and brain and accordingly, activities of selenoenzymes as well (158). Another study confirmed that on the mRNA level, Sepp1 KO mice on standard lab diets have been reported to have decreased GPx1, GPx4, and SelW in whole brain tissue corroborating with enzyme activity and Se levels previously reported (104) .

Genetic deletion of Sepp1 in mice result in a multitude of neurological impairments such as neurological seizures and movement disorders particularly when placed on restricted selenium diets (8,101,159). $Sepp1^{-/-}$ mice on 0.1 mg Se/kg diet have very low selenium concentrations in the brain and testis, with severe pathophysiological consequences in each tissue (102). Selenium concentrations are very low in fetal $Sepp1^{-/-}$ mice as well. Selenium levels in brains of these $Sepp1^{-/-}$ mice were reduced to 43% but did return to $Sepp1^{+/+}$ levels with a high selenium supplementation of 2 mg Se/kg (101). In testes however, selenium levels were decreased to 19% and did not increase even with the same high selenium supplementation suggesting targeted priority of Sepp1 towards the brain (101). $Sepp1^{-/-}$ mice fed a Se inadequate diet of less than 0.1 mg/kg, originally generated by the Burk lab, exhibited an abundance of deficiencies compared to their wild type counterparts including reduced weight, smaller body size, poor motor coordination development, and

strikingly reduced fertility (101). A study by the Burk lab showed genetic deletion of Sepp1 did not alter hepatic Se levels when maintained on a Se adequate diet (0.25 ppm) (101). When Sepp1$^{-/-}$ mice were maintained on a diet with less than adequate Se, below 0.1 mg/kg, liver Se levels were then increased (101). Kidney selenium levels were reduced to 76% of normal levels and did increase to normal levels upon a selenium adequate diet of 0.25 mg/kg (101). Deletion of Sepp1 causes increased excretion of selenium in the urine and, as a result, decreases whole-body selenium (103,160). Sepp1$^{-/-}$ mice on a selenium deficient diet of 0.1 mg/kg developed spasticity and abnormal movements, performing poorly on motor coordination tests such as the rotorod and pole climb, whereas this diet provided sufficient selenium for wild type mice to perform normally on these tests (161).

The results of the study indicate that the deletion of Sepp1 results in irreversible brain damage (103). Sepp1$^{-/-}$ mice on an adequate (0.25 ppm) or high Se (1 ppm) supplemented diet show increased survival rate, improved neuromotor function, resumed weight gain, stabilized neurological function but not to normal abilities (161). However, brain Se concentration did not increase (161) and importantly, synaptic plasticity deficits as shown by lack of LTP in these mice remain (162). ApoER2 is one of the known receptors to which Sepp1 binds. ApoER2$^{-/-}$ mice on a Se deficient diet showed similar neurological deficits to those of Sepp1$^{-/-}$ mice (103). The study suggests that interruption of Se supply evidenced by decreased Se uptake to the brain leading to neurological deficits was due to impairment to the Sepp1-ApoER2 pathway (103). Sepp1$^{-/-}$ mice are deficient in long term potentiation (LTP), a model for cellular learning and memory (162). The current data underscore the importance of Sepp1 in neurological function and a need to further understand the function of Sepp1.

Selenoprotein M

SelM is an endoplasmic reticulum (ER) protein that is abundantly expressed in the brain relative to other tissues (39,96), and has been reported to have neuroprotective properties. SelM is expressed in several regions, including the CA2 region of the hippocampus- specifically CA2/CA3 regions, medial septum, somatosensory cortex, hypothalamus, thalamic reticular nucleus, ventral tegmentum,

and Purkinje layer of the cerebellum (100). Some other brain regions that were noted to have high levels of SelM expression included the ventral tegmental area, red nucleus, medial septum, and several structures associated with auditory processing (cochlear nucleus, olivary complex, lateral lemniscus) (100). Neuronal signaling is complex and involves many synchronous events. The following neuronal signaling processes coincide with areas where SelM is expressed and is briefly described here. The sensory information routed to the cortex via the thalamus must be prioritized. For instance, stimuli that signal danger or opportunity must be filtered and processed in a specific order. These processes are carried out via the thalamic reticular nucleus, between the thalamus and cortex (163) This represents an inhibitory interface, or attentional gating, which regulates the flow of information between the thalamus and cortex. Studies show that the thalamic reticular nucleus is involved in higher cognitive functions, including learning, memory, and spatial cognition (163). The medial septal area receives reciprocal connections from the hippocampus and cerebral cortex, cingulate gyrus and thalamus (164). The septal nuclei play a role in reward and reinforcement along with the nucleus accumbens (165). The cingulate gyrus is situated in the medial aspect of the cortex and receives inputs from the thalamus, and projects to the entorhinal cortex, which is involved with emotion formation and processing, learning, and memory (166).

In one of the first studies to characterize SelM in neuronal function, Reeves et al. demonstrated that SelM modulates calcium release from intracellular ER stores during oxidative stress in response to H_2O_2 in HT22 hippocampus cells and C8-D1A cerebellum cell cultures and has a protective effect against oxidative stress in neuronal cells (2). Overexpression of SelM has been shown to prevent H_2O_2 induced oxidative stress while shRNA knockdown of SelM in HT22 cells and primary cortical cultures led to decreased viability and apoptotic cell death (2,8,97). Another study utilized a chicken model to confirm that SelM is also present in the chicken brain and a Se deficient diet resulted in downregulation of SelM mRNA and protein as reported in studies using rodent models (167). A study using an Alzheimer's disease mouse model having a mutant form of presenilin-2 resulted in suppression of SelM expression (3). Mutations in the presenilin-2 gene cause autosomal-dominant early-onset Alzheimer's disease (168–170). A more recent study found that SelM interacts with Galectin-1 (Gal-1) using a

yeast-2-hybrid system and confirming their results various other tools such as fluorescent resonance energy transfer (FRET), glutathione-S-transfer (GST) pull-down and co-immunoprecipitation assays (171). Gal-1 is an endogenous mammalian lectin (171). This soluble protein is present both inside and outside cells, has both intracellular and extracellular functions, and is widely expressed in mouse brain neurons, neural stem cells, and neuroblasts (171,172). It regulates neural cell fates, such as cell proliferation, differentiation, and death (171,173), essential for neurogenesis and in the recovery from brain damage (171,174,175) and also plays important roles in adult neural stem cells under both physiological and pathological conditions (171,176). Gal-1 deficiency led to attenuated proliferation of neural progenitors in the hippocampal dentate gyrus (DG) (171). The study posits that the neuroprotective action of SelM may be indirectly mediated by Gal-1. A study using a transgenic rat model, CMV/hSelM Tg rat, showed that SelM overexpression, and selenium treatment to induce a high antioxidant activity, affects global gene expression in the brain cortex contributing towards increased selenium bioavailability in the brain. One of the eight proteins the study found to be modulated by SelM overexpression was synaptotagmin-15 (SytXV), known to be a Ca^{2+} sensor that regulates Ca^{2+}-dependent membrane trafficking, including endocrine exocytosis (177–179), synaptic vesicle exocytosis (179–181), and neurotransmitter release (181,182). Tg rats also had a higher rate of decrease in γ-secretase activity. Part of the presenilin complex, γ-secretase plays an important role in the production of Aβ-42 peptide in the pathogenesis of AD (179).

Many members of the selenoprotein family are known to have multiple functions and also roles in metabolism. In a follow-up study, Pitts and Reeves et al., further described that the genetic deletion of SelM in mice resulted in increased adiposity leading to obesity which corroborated with the enriched SelM levels observed in the hypothalamic nuclei, an area involved in energy metabolism (100). The study also showed $SelM^{-/-}$ did not exhibit any apparent neurobehavioral deficits in regards to general locomotion and anxiety, motor learning and coordination, and spatial learning and memory (100). However, the $SelM^{-/-}$ mice did exhibit increase in body weight and adiposity suggesting that absence of SelM may effect metabolic function (100). $SelM^{-/-}$ male mice had elevated fasting insulin levels while $SelM^{-/-}$ female mice were

comparable to their wild-type counterparts. The study also revealed a gender difference in body composition and other metabolic measures. However, in this study we will show explicit evidence that SelM participates in regulating learning and memory by investigating its role in maintaining normal synaptic physiology.

Selenoprotein P and Selenoprotein M are associated with Alzheimer's disease pathology. Alzheimer's disease (AD) is the most common cause of dementia, afflicting more than 5 million people in the United States alone. This debilitating disease involves the progressive loss of cognitive and behavioral function. AD is the sixth leading cause of death and is projected to affect 1 in 85 people in the next 40 years (183,184). A new case of AD is expected to develop every 33 seconds by 2050. In 2013, Alzheimer's was estimated to cost the nation $203 billion. This number is expected to rise to $1.2 trillion by 2050 (184). The hallmarks of Alzheimer's disease are the characteristic extracellular plaques consisting of the amyloid beta (Aβ) protein and the presence of intracellular neurofibrillary tangles (183,184). The Aβ peptide contains either 40 or 42 amino acids, and is cleaved from the amyloid precursor protein (APP) by way of the β or γ secretases (185,186).The disease is also marked by a significant decrease in neurons, synapses, and thus synaptic plasticity. AD is also associated with lipid, protein and nucleic acid oxidation and neuronal death (187). Chronic, elevated oxidative stress precedes loss of neurological function and cell degeneration in AD and other neurodegenerative diseases (117).

Strong evidence shows that many selenoproteins have properties that are beneficial in preventing human disease. Se has been proposed for treatment and prevention of AD and is being investigated in clinical trials. Published and preliminary data demonstrate that selenoproteins such as Sepp1 and SelM are involved in AD and undoubtedly required for normal brain function. Sepp1 has several protective properties, including binding of metals such as zinc, copper and mercury, and a redox domain reported to exhibit antioxidant properties. Sepp1 protein expression is increased in AD brain compared to normal brain. Previous work from Bellinger et al., has also demonstrated that Sepp1 associates with amyloid plaques and neurofibrillary tangles in the choroid plexus of post-mortem Alzheimer's disease (AD) brain suggesting

potentially neuroprotective properties (2,39). Knockdown of Sepp1 increases neurotoxicity from Aβ peptides, supporting the protective role of this protein (1). Sepp1-deficient mice have impaired spatial learning and dramatic impairments in synaptic signaling and plasticity. ApoE is a ligand for ApoER2 and other LRP receptors. The ApoE4 allele type is a genetic risk factor for AD (188). Megalin has a role in clearing Aβ from brain through the choroid plexus (189), and has polymorphisms that may also increase risk of AD (190,191). Both ApoER2 and megalin null animals are deficient in brain Se. Sepp1 is able to bind transition metal ions and modulate the Zn^{2+}-mediated Aβ aggregation, ROS production and neurotoxicity (192).

This study will further explore the role of Sepp1 in synaptic plasticity independent of its function in transporting selenium. Sepp1 is implicated in maintaining normal neurological function but its role in the brain and neurodegeneration, independent of Se transport remains unclear.

SelM function has also been linked to neurodegenerative disease models and may have a role in preventing Alzheimer's disease pathology. A previous study of mice overexpressing a human presenilin-2 (PS2) mutation associated with AD revealed suppressed SelM expression (3). This PS2 mutation results in an autosomal dominant familial form of early onset AD (185). The PS2 consists of two missense mutations in γ-secretase causing over-production of the amyloid-β peptide resulting in increased $Aβ_{42}$ in the plasma (170,185,193,194). SelM activates the ERK but not MAPK pathway involving p38 and JNK, to attenuate alpha/gamma-secretase-mediated proteolysis and Tau phosphorylation to protect brain function (195). In a study utilizing selenium supplementation of SelM overexpressing transgenic rats the ERK signaling pathway was significantly increased in response to selenium treatment, and unchanged in non-transgenic rats (195). SelM is capable of binding to transition metal ions and modulating the Zn^{2+}-mediated Aβ aggregation, ROS production, and neurotoxicity (192). Most recently, overexpression of SelM has been shown to inhibit beta amyloid beta peptide ($Aβ_{42}$) when cotransfected together in HEK-293T cells (196). Despite recent characterizations and discoveries, the function of SelM, particularly in the brain, remains largely unknown.

Understanding how Sepp1 and SelM exert neuroprotective effects is vital in revealing the important association of these selenoproteins and their regulatory roles in neurodegenerative disorders such as Alzheimer's disease. Elucidating the neurological function and neuroprotective mechanisms of selenoproteins would facilitate their application towards a therapeutic treatment of this debilitating neurodegenerative disease that has yet to have a cure.

Synaptic Plasticity

Synaptic plasticity refers to the process in which the connections between neurons otherwise known as synapses, strengthens or weakens over time in response to increase or decrease in activity (197). In 1949, Donald Olding Hebb first proposed synaptic plasticity as a mechanism for learning and memory. Two main forms of synaptic plasticity were proposed, long-term potentiation and long-term depression. The hippocampus, an important region involved in memory, learning, and neurogenesis, has been shown to be one of the first brain regions affected by AD. A multitude of factors are involved in regulating normal synaptic plasticity. The dentate gyrus of the hippocampal formation is also involved in adult neurogenesis (198). The subgranular layer (SGL) of the dentate gyrus is partially comprised of progenitor cells that proliferate, differentiate, and give rise to young neurons that can become integrated into existing neuronal circuits (198). Under physiological conditions, hippocampal-dependent learning has been linked to hippocampal neurogenesis, whereas deficits in adult hippocampal neurogenesis have been shown to correlate with disturbances in spatial learning and memory (198).

Different forms of synaptic plasticity regulate different types of memory formation and learning, categorized as declarative (explicit) and non-declarative (implicit). Declarative memory, includes the memories of facts and events. Non-declarative memory, include the memories for skills and habits, a phenomenon called priming, simple forms of associative learning such as those observed in classical conditioning (i.e Pavlovian conditioning), and simple forms of non-associative learning such as habituation and sensitization (199). Long-term as opposed to short-term memories involve changes in protein synthesis, gene regulation, and often long-term memories

involve structural modifications. Studies have shown that changes can occur just 24 h after sensitization training (200,201). Neurons from trained animals exhibit greater number of branches and an increased number of synaptic varicosities than the neuron from the untrained animal (202).

In the central nervous system (CNS), synaptic strength and activity-dependent synaptic plasticity are mainly dependent on the function of N-Methyl-d-aspartate receptors (NMDAR). NMDARs consist of two requisite GluN1 subunits and two additional GluN2 or GluN3 subunits that confer the specific properties of the receptor (203). NMDAR activity is partly regulated by phosphorylation and dephosphorylation on tyrosine residues in the cytoplasmic domains of the NMDA receptor NR2A or NR2B subunits by SFK (204,205), and phosphorylation increases ion conductance (205–209). These receptors are expressed on the postsynaptic side of excitatory synapses and regulate both synapse formation during development as well as synaptic plasticity in the adult brain by controlling Ca^{2+} flux into the neurons (205,210,211). These are one the mechanisms of action that occur in long-term potentiation (LTP) exhibited by the Schaffer collateral-CA1 region of the hippocampus (212,213).

LTP is a form of synaptic plasticity thought to be involved in declarative memory, in which high frequency stimulation of neural pathway induces signal transmission that results in long-lasting increased synaptic efficacy between neurons (214). LTP is a mechanism necessary to store a long-term memory. LTP, originally discovered in rabbit hippocampus, can be observed in other brain regions including but not limited to the cerebellum, amygdala, and cerebral cortex (215). Different regions of the brain exhibit variations of LTP and can be dependent on different molecules. LTP in the CA1 region of the hippocampus has been the primary model by which to study the cellular and molecular basis of memory (213). There are many varieties, but a typical protocol to induce LTP can consists of two trains of high frequency stimulation, also referred to as the tetanus, (HFS: 100 Hz, 1sec) spaced 20 s apart (216) to afferent nerves resulting in augmentation of the postsynaptic neuron primarily in two manners . In hippocampal slices of wild type mice this stimulation protocol reliably induces LTP, characterized first by a transient and highly robust post-tetanic increase in the slope of the fEPSP that is followed by a sustained and less robust increase (216). Others forms of LTP depend on

metabotrophic glutamate receptor (mGluR), cyclic adenosine monophosphate cAMP or other molecules (217). Both LTP and long-term depression (LTD) are necessary as LTD prevents saturation of synapses due to continuous stimulation leading to loss of selectivity and as well as to prevent a positive feedback loop between synapse network activity, help maintain neural homeostasis (218,219). Briefly, LTD occurs in many regions of the brain, but has been studied mostly in the hippocampus and cerebellum (220). LTD is the process in which synaptic efficacy is reduced (220).

Paired-pulse facilitation (PPF) is a form of short-term plasticity. When two pulses at a short interpulse interval are given to the afferent pathway, the postsynaptic response to the second stimulus is increased when compared with the first response (162). This phenomenon is understood to be due to residual calcium in the presynaptic terminal that facilitates neurotransmitter release upon the second stimulation, resulting in the subsequent increase in the post synaptic response (162,221). PPF can provide information regarding the loci of the LTP expression, whether changes in the response is mediated presynaptically and most likely not postsynaptically. When LTP expression occurs at a presynaptic loci, then it may alter PPF potentiation. Changes in PPF are inversely correlated to initial PPF responses. A smaller initial PPF is associated with an increase in PPF while a larger initial PPF was associated with a decrease in PPF with LTP (222). Alterations in PPF response within the same time course as LTP also suggest it is input specific and not due to nonspecific effects of high-frequency stimulation, changes in inhibition, active postsynaptic currents or their nonlinear summation, and PPF changed with the same time course as LTP (222).

Conclusion

In this study, we will focus on hippocampal NMDA receptor dependent LTP and the roles of selenoproteins P and M in maintaining proper synaptic plasticity. We elucidate that Sepp1 has a critical role in the brain, regulating hippocampal dependent synaptic plasticity, independent of its known function of selenoprotein distribution to all tissues in the body. Further investigation is important for elucidating the functions and mechanisms of selenoproteins to understand how altering levels of Se and different

selenoproteins may affect neurological function and neurodegenerative conditions such as AD.

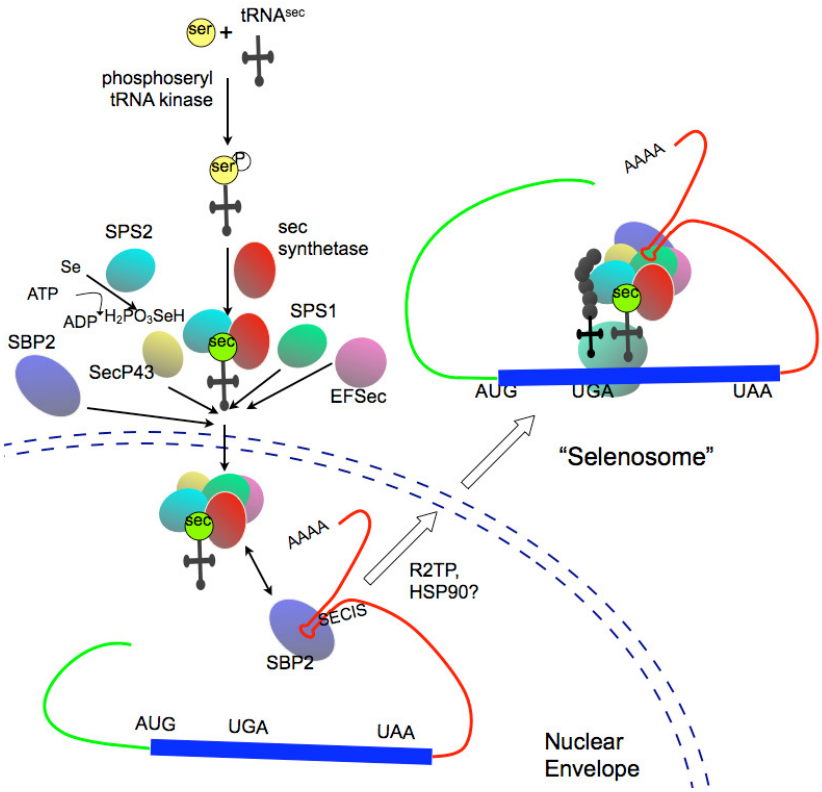


Figure 1. Selenoprotein biosynthesis pathway. Phosphorylation of Se by selenophosphate synthetases (SPS) is used to synthesize selenocysteine (Sec) from serine directly on the $tRNA^{Sec}$ by the enzyme Sec synthetase. $tRNA^{Sec}$ is transported to the nucleus with many bound cofactors. The protein selenoprotein binding protein 2 (SBP2) binds to the selenocysteine insertion sequence (SECIS) element in the 3' UTR of selenoprotein messages, engaging the $tRNA^{Sec}$ complex along with bound cofactors. The assembled complex is transported from the nucleus for translation to protein (8,122). Schematic adapted from Bellinger et al., 2009 (8).

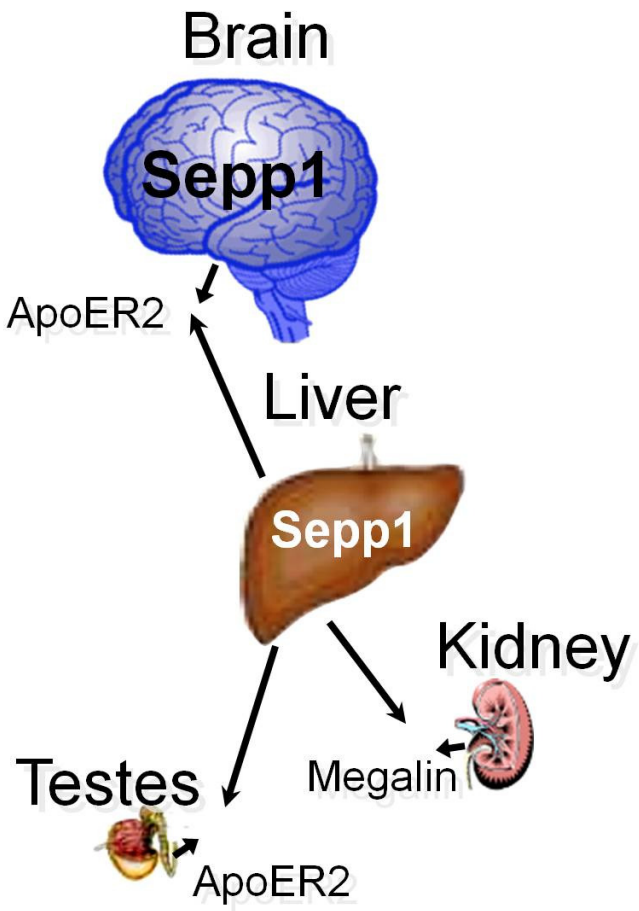
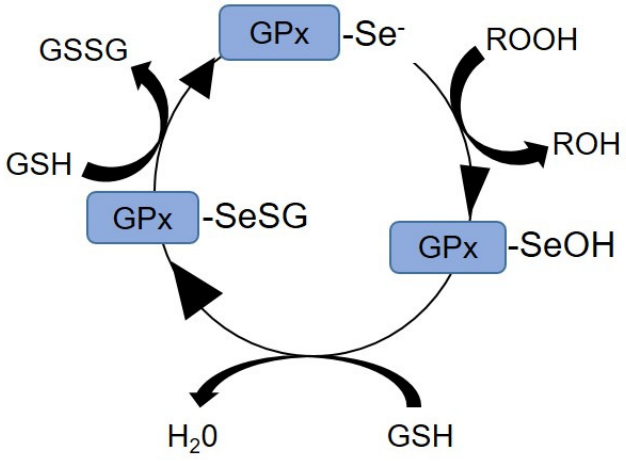


Figure 2. Selenoprotein P biosynthesis pathway. Sepp1 is principally synthesized in the liver, and secondarily in smaller amounts in the brain. Sepp1 is thought to transport selenium to other tissues in the body. Sepp1 binds to the low-density lipoprotein receptor ApoER2 in brain and testes and Megalin in kidneys.



$$2GSH + H2O2 \rightarrow GS–SG + 2H2O$$

1. $RSeH + H2O2 \rightarrow RSeOH + H2O$
2. $RSeOH + GSH \rightarrow GS\text{-}SeR + H2O$
3. $GS\text{-}SeR + GSH \rightarrow GS\text{-}SG + RSeH$

Figure 2. Glutathione Redox Cycle

GSH represents reduced monomeric glutathione, and GS–SG represents glutathione disulfide. The selenol of a selenocysteine residue is oxidized by hydrogen peroxide. This process yields a derivative with a seleninic acid (RSeOH) group. The selenenic acid is then converted back to the selenol with a reaction beginning with GSH to form the GS-SeR and water (58,223). A second GSH molecule reduces the GS-SeR intermediate back to the selenol, releasing GS-SG as the by-product. Glutathione reductase then reduces the oxidized glutathione to complete the cycle: GS–SG + NADPH + H+ → 2 GSH + NADP+ (58,223).

Table 1. Mammalian selenoproteins: localization and functions

Selenoprotein	Localization	Function	References
15 kDa selenoprotein (Sep15)	ER	Trx-like fold, regulated by ER stress, interacts with UDP-glucose:glycoprotein glucosyltransferase, potentially involved in glycoprotein folding in the ER	(57,98,224, 225)
Thyroid hormone deiodinase 1 (DI1, Dio1)	Plasma membrane	removes iodine from the outer ring of T4 in the thyroid to produce plasma T3–catalyzes deiodination and thus inactivation of T3	(226,227)
Thyroid hormone deiodinase 2 (DI2, Dio2)	ER	converts T4 to T3 locally in tissues including pituitary, brown fat, and brain	(228)
Thyroid hormone deiodinase 3 (DI3, Dio3)	Plasma membrane	catalyzes deiodination of T4 to T3 in peripheral tissues	(227,229)
Glutathione peroxidase 1 (GPx1)	Cytosol	GSH-dependent detoxification of H_2O_2 (highly expressed in liver, kidney, erythrocytes), Se deficiency leads to nonsense mediated decay of GPx1 mRNA	(230)
Glutathione peroxidase 2 (GPx2)	Cytosol	GSH-dependent detoxification of H_2O_2 (highly expressed in the epithelium, particularly in the intestine and lung)	(231,232)
Glutathione peroxidase 3 (GPx3)	Extracellular, CSF	GSH-dependent detoxification of H_2O_2 (synthesized predominantly by kidneys and secreted to plasma), protects against oxidative stress in thyroid, involved in protection of cardiovascular system through modulation of nitrous oxide levels	(233,234)
Glutathione peroxidase 4 (GPx4, PHGPx)	Cytosol, mitochondria, nucleus (testis-specific)	has cytosolic, nuclear and mitochondrial isoforms–protects lipids from H_2O_2-mediated oxidation, structural protein in sperm, sensor of oxidative stress, and pro-apoptotic signals	(235)

Glutathione peroxidase 6 (GPx6)	Cytosol	GSH-dependent detoxification of H_2O_2 (enriched in the olfactory epithelium)	(232)
Selenoprotein H (SelH)	Nucleus	Trx-like fold, protects cells from H_2O_2, involved in transcription, increases mitochondrial biogenesis and CytC production–AT-hook family protein. In response to redox flux, facilitates synthesis of genes responsible for de novo GSH synthesis	(80,85,236)
Selenoprotein I (SelI)	Membrane	may be involved in phospholipid biosynthesis	(39)
Selenoprotein K (SelK)	ER membrane	modulates Ca^{+2} influx that affects immune cell function–component of ERAD; involved in the Derlin-dependent ERAD of glycosylated misfolded proteins; involved in proteolytic modulation in macrophage activation via calpain/calpastatin system	(88)
Selenoprotein M (SelM)	ER	Trx-like fold, thiol-disulfide oxidoreductase, protects neurons from oxidative stress; may be involved cytosolic calcium regulation, involved in body weight regulation, and energy metabolism	(237)
Selenoprotein N (SelN,SEPN1,SelN1)	ER membrane	expressed in skeletal muscle, heart, lung, and placenta; regulates redox state of the intracellular calcium-release channel, ryanodine receptor (RyR); affects Ca^{+2} homeostasis, SelN gene mutations cause congenital myopathy such as multiminicore diesease	(238,239)
Selenoprotein O (SelO)	Mitochondria	has Cys-XX-Sec motif, largest mammalian selenoprotein, widely distributed throughout many tissues, important in redox function, expression not greatly affected by Se dificiency status, Sel O deficiency leads to impairment of chondrocyte cell differentiation, viability, and proliferation	(39)

Selenoprotein P (SelP, Sepp1)	Extracellular	facilitates Se transport to peripheral tissues, primarily the brain and testes particularly during low Se status, and antioxidant function; important for sperm and oogenesis, and synaptic plasticity; Sepp1 deletion leads to severe neurological conditions and infertility	(157,238,240)
Selenoprotein R (SelR, MsrB1, Selx1)	Cytosol	reduces methionine-R-sulfoxide residues in proteins to methionine; Se and Zinc containing protein; protection against oxidative stress and/or in redox regulation of cellular processes.	(241)
Selenoprotein S (SelS, SEPS1, Tani, VIMP, and SELENOS)	ER membrane	upregulated upon treatment with pro-inflammatory cytokines and glucose deprivation– ERAD component; interacts with other proteins via coiled coil region; provides intracellular membrane transport and maintenance by anchoring protein complexes to ER membrane; SelS is disregulated in diabetic patients	(239,242)
SPS2	Cytosol	de-novo synthesis of selenophosphate necessary for Sec biosynthesis, all selenoproteins including itself, maybe also involved in Cys synthesis	(243,244)
Selenoprotein T (SelT)	ER and Golgi	Trx-like fold, redox regulation–plays a role in cell adhesion and calcium mobilization	(245)
Thioredoxin reductase 1 (TR1, Txnrd1)	Cytosol	reduces the oxidized form of cytosolic thioredoxin–has at least 6 isoforms differing in N–terminal sequences; genetic deletion is embryonic lethal	(246,247)
Thioredoxin/ glutathione reductase (TGR, TR2, Txnrd3)	Cytosol	has a glutaredoxin domain, catalyzes a variety of reactions, specific for thioredoxin and glutaredoxin systems–expressed in spermatids, genetic deletion is embryonic lethal	(248)
Thioredoxin reductase 3 (Txnrd2, TR3)	Mitochondria	reduces the oxidized form of mitochondrial thioredoxin and glutaredoxin 2, specifically expressed in the testes; regulated by Wnt signaling in the intestinal epithelium	(249)

Selenoprotein V (SelV)	Cytosol	Trx-like fold–unknown function–expressed in spermatids	(85)
Selenoprotein W (SelW)	Cytosol	Trx-like fold - has thioredoxin-like function; expressed in skeletal muscle-may be important in muscle growth , heart (except rodents), spleen, and brain, highly dependent on adequate dietary Se and Sepp1 levels, putative antioxidant role- responds to stress, involved in cell immunity, specific target for methylmercury	(250)

(Adapted from Kasaikina et al., 2012 (57))

CHAPTER II

GENETIC RESCUE OF SEPP1 IN SELENOPROTEIN KNOCKOUT MICE

Abstract

Genetic deletion of Sepp1 results in multiple neurological impairments, male infertility and sperm morphological defects (10). We hypothesized that utilizing a novel application of the Cre-*LoxP* system could restore Sepp1 gene expression in Sepp1$^{-/-}$ mice using the *LoxP* start codon. In this study, Sepp1 mice were bred with mice transgenic for the cytomegalovirus-Cre (CMV-Cre) gene, excising the floxed neo^{r} construct in the Sepp1$^{-/-}$ mice. Our data show that this approach produced viable progeny of the systemic Sepp1$^{r/r}$ CMV+ (rescue) mice that express the CMV-Cre driven Sepp1 gene in all tissues. The progeny had restored neuromotor function, the spatial learning and memory deficits were negated, and sperm morphology and mobility appeared normal and comparable to wildtype mice. This unique genetic rescue technique can be employed to investigate the localized function of Sepp1 in specific cells, independent from its global function, and provides a proof-of-concept for a technique to restore expression of a target gene to specific cells in a knockout mouse model.

Introduction

Sepp1 is a critically important selenoprotein due to its well-known function in transporting selenium and facilitating biosynthesis of other selenoproteins. Sepp1$^{-/-}$ mice develop severe neurological impairment as mentioned, some of which can be improved by dietary Se supplementation. However, synaptic plasticity impairments remain despite dietary Se supplementation (216,251). A study by Schweizer et al. showed that targeted inactivation of the Sepp1 gene led to hepatocyte-specific inactivation of selenoprotein biosynthesis, thereby reducing plasma and kidney selenium levels similarly to Sepp$^{-/-}$ mice, but did not result in neurological impairment (252). Furthermore, brain and testis are Se privileged tissues under Se deficient conditions (253). Taken together with other

current understanding of Sepp1 functions, mounting evidence suggests a physiological role of locally expressed Sepp1 in the brain and potentially other tissues.

Sepp1$^{-/-}$ mice fed a selenium inadequate diet of less than 0.1 mg/kg exhibited an abundance of deficiencies compared to their wild type counterparts. These included reduced weight, smaller body size, and significantly reduced fertility. These mice also developed severe spasticity and abnormal movements, and performed poorly on motor coordination tests such as the rotarod and pole climb, whereas the wildtype counterparts appeared unaffected on this diet (101,161).

The Sepp1$^{-/-}$ mice were designed with a reverse-orientation neo^{r} cassette flanked by LoxP sites, inserted into the second exon, 9 bases downstream of the start codon (Fig. 4). This was introduced by electroporation into 129S9/SvEvH-derived embryonic stem (ES) cells (101), and these ES cells were subsequently injected into C57BL/6 blastocysts. The resulting chimeric males were bred with C57BL/6J females. The stop codons present in both LoxP sites prevent translation of SEPP1. The neo^{r} containing construct effectively disrupts Sepp1 expression.

Based on sequence analysis of the Sepp1 targeting construct, we predict that introduction of a Cre recombinase transgene to the Sepp1$^{-/-}$ mice would excise the floxed neo^{r} containing construct, however, preserving a start codon in the LoxP site in frame with the open reading frame in the SEPP1 gene. Translation from the new start codon would result in changes of only a few amino acids at the N-terminus of Sepp1, which contains a signal peptide for secretion. The gene is still functional with these minor changes (Fig.3B-C) as the signal peptide is cleaved from the mature protein, restoring the wild-type sequence. Successful implementation of this method will allow for applying this to restrict gene expression to specific cells.

Materials and Methods

Animals

Animals were provided food and water ad libitum per University of Hawaii veterinary protocol. All animals in this study were maintained on diets containing adequate Se (~0.25 ppm). Animals were kept on a 12-h light cycle and group housed during breeding and rearing. Prior to and during behavioral testing, animals were individually housed in polycarbonate cages. Each cage was provided food, water, and a layer of bed-o-cob (corn cob) bedding (Newco Distributers). All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Generation of Sepp1$^{-/-}$ and Sepp1$^{r/r}$ CMV-Cre

Sepp1$^{-/-}$mice were obtained from the laboratory of Dr. Raymond Burk at Vanderbilt University. Mutant mice were backcrossed to C57BL/6J for at least 10 generations before arriving in our lab and were bred with our C57BL/6J colony to ensure congenic strains (254). As male Sepp1 mice are infertile (101), Sepp1$^{+/-}$mice were used for breeding resulting in littermate Sepp1$^{-/-}$,Sepp1$^{+/+}$, and Sepp1$^{+/-}$pups. Sepp1 whole body genetic rescue mice (Sepp1$^{r/r}$ CMV+) were generated by breeding Sepp1$^{-/+}$ mice to B6.C-Tg(CMV-cre)1Cgn/J (Jackson Labs). The commonly used cytomegalovirus (CMV) promoter provides strong and constitutive expression in many cell types. Sepp1$^{r/r}$ CMV+ rescue mice were generated by breeding Sepp1$^{+/-}$ mice to CMV-Cre expressing mice, B6.C-Tg(CMV-cre)1Cgn/J. Sepp1 $^{-/-}$ mice, originally generated by the Burk lab, were designed with a reverse-orientation neo^{r} cassette flanked by LoxP sites, inserted into the second exon, 9 bases downstream of the start codon (Fig. 4). The neo^{r} containing construct effectively disrupts Sepp1 expression by interrupting the reading frame of the Sepp1 sequence thus preventing the Sepp1 gene to be functional. The genetic deletion of Sepp1 results in a hosts of impairments including male infertility (10). Confirmation of the expected recombination of Sepp1 was carried out by polymerase chain reaction (PCR) of extracted genomic DNA from mouse tails using specific primers to amplify a 151-bp product in the targeted region present in the wild type gene (forward-ACCTCAGCAATGTGGAGAAGCC, reverse-TGCCCTCTGAGTTTAGCATTG), and 472-bp and 224-bp products specific for the knockout allele and floxed gene, respectively

(forward-ACCTCAGCAATGTGGAGAAGCC, reverse-GATGATCTGGACGAAGAGCATCA). Products were analyzed on 1.5% DNA agarose gels, and SYBR® Safe DNA Gel Stain (InVitrogen) was detected by UV imaging to confirm genotype.

Animal Behavior

Age matched adult female and male Sepp1$^{+/+}$, Sepp1$^{r/r}$ CMV+, or Sepp1$^{-/-}$ mice 12 to 24 weeks of age (age matched for each assay) were evaluated for neuromotor and neurobehavior effects using typical behavior paradigms as follows.

Vertical Pole Test

Mouse locomotor function involving the cerebellum, motor cortex, and basal ganglia (255) was evaluated using a pole test. Mice were placed on the top of a pole with heads oriented upward and parallel to the pole. Mice were given 60 s to perform this task. After 2 days of 4 training trials per day, the time taken to invert and face downward and the total time to descend were recorded in 4 trials on the third day, in which the best turn and descent time was used for analysis between genotypes.

Stride test

A stride test, modified from Fernagut et al. (256), was used to measure deficiencies in gait and motor ability. Paw prints were obtained by applying ink to the hind limb paws of the mice before placing them on graph paper in a narrow runway. Bright lighting was used to encourage the mice to walk toward a dark enclosure at the opposite end of the runway. Length of stride for each paw and width of strides were measured from the resulting footprints.

Morris water maze

Hippocampal-dependent spatial learning and memory was assessed using a Morris Water Maze (MWM) assay (216). Mice were placed in a large circular pool of opaque water heated to 24℃. Nontoxic, water based paint was added to achieve opacity. Visual cues were placed on walls to provide mice with spatial orientation and reference points.

The time required to escape from water onto a hidden platform was measured. The total time required for mice to swim to a visual platform (60 s maximum) was determined prior to the training days. Each mouse was given 60 s to find the visible platform or after this period, the mouse would be removed from water and placed on the platform for 15 s. During the 8 days of training of 4 trials per day, the platform was submerged and the total time (60 s maximum) for mice to find the hidden platform was recorded. On the ninth day, the platform was removed for the probe trial. The total time spent in each quadrant and the number of platform crossings were monitored over a 60 s period. Sepp1$^{-/-}$ mice MWM data were obtained in a different experiment in which all experimental procedures and parameters were the same, and kindly provided by Dr. Matthew Pitts. Sepp1$^{-/-}$ mouse data were included only as an example for comparison of the behavioral deficits previously reported in Sepp1$^{-/-}$ mice (113,216).

Western Blot

GPx4 protein expression in testes and epididymides of age matched Sepp1 WT, Sepp1$^{-/-}$, and Sepp1 rescue mice were measured by western blot. Tissues were lysed with CelLytic MT (Sigma Aldrich Co) according to the manufacturer's instructions. Protein lysates were resolved by SDS-PAGE separated on a 10–20% gradient Tris-HCl Criterion Precast gel (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride (PVDF). For detection of GPx4 expression in testes, membranes were incubated in rabbit GPx4 polyclonal antibody (AbFrontier) diluted 1:2000, and for epididymides, GPx4 polyclonal antibodydiluted 1:5000 (Epitomics) in 1:4 Odyssey blocking solution in PBS (LI-COR Biosciences) for 90 min at RT. Following washes in PBS (5 X 5 min), primary antibodies were detected with LI-COR near-infrared fluorescent secondary antibodies in 1:4 blocking solution in PBS for 45 min followed by washes as described above. Subsequently blots were developed using β-actin (Sigma Aldrich Co.) as a loading control. Protein was detected using the Odyssey® Infrared Imaging System (LI-COR Biosciences). Scanning and analysis were performed with LI-COR Odyssey software.

Statistical analysis.

Statistical analysis was performed with GraphPad Prism software. Sample size were determined through power analysis (257,258). Interaction between genotypes and sex was ascertained by Student's t-test and two-way analysis of variance (ANOVA) with Bonferroni's posthoc test for multiple comparisons. To determine genotype differences between experimental groups in the water maze training and quadrant entries in the probe trial, repeated-measures ANOVA with Bonferroni's posthoc test were used. Statistical significance was defined as having $p<0.05$ for all statistical tests.

Results

Genetic rescue of Selenoprotein P expression using Cre-recombinase.

Sepp1$^{+/-}$ littermates were used for generating the rescue mice due to the infertility of Sepp1$^{-/-}$ males. Breeding Sepp1$^{+/-}$ mice with the CMV-Cre resulted in recombinase expression in all cells, and the resulting recombination excised the neo^{r} construct in the Sepp1$^{-/-}$ mice. Sepp1 genetic recombination in the Sepp1$^{r/r}$ CMV+ was confirmed with PCR genotyping, by identifying an amplified 224-bp product specific for the recombined gene in comparison to the 151-bp product in the targeted region present in the wild type and a 472-bp product for the knockout allele (Fig. 4A). A start codon in the remaining single LoxP site was left in frame with the Sepp1 gene, thus resulting in translation of a form of Sepp1 with a minor mutation in the Sepp1$^{r/r}$ CMV+ mice (Fig. 4B-C). The male Sepp1$^{r/r}$ CMV+ progeny sired pups, indicating that the Sepp1 gene was restored and functional.

Neuromotor behavior and gait impairments are recovered in Sepp1$^{r/r}$ CMV+ mice.

Sepp1$^{-/-}$ mice have severe motor impairments encompassing irregular gait patterns described to include dragging of the limbs and uneven strides as well as motor coordination deficits (159,161). We used the pole and stride tests to assess genetic restoration in the Sepp1$^{r/r}$ CMV+ mice. Motor coordination and general locomotor function of Sepp1$^{r/r}$ CMV+ mice were indistinguishable from those of the wild type group. The rescue mice in comparison to the wild type group exhibited no significant differences in the total time to descend the pole, whereas the Sepp1$^{-/-}$ mice had significantly decreased coordination and locomotion as shown by the greater total amount of time Sepp1$^{-/-}$ mice took to descend to the bottom of the pole (Fig. 5). One-way ANOVA showed an effect of genotype on the time taken to descend (**P=0.0086 for Sepp1$^{r/r}$ CMV+, Bonferroni's post hoc test **P<0.01) and turn time (**P=0.0024, with Bonferroni's posthoc test **P<0.01 for Sepp1$^{r/r}$ CMV+ and *P<0.05 for Sepp1$^{+/+}$), n= 6 per group.

We subjected the mice to a stride test to determine whether the irregular gait and ataxia seen in Sepp1$^{-/-}$ mice was recovered in the Sepp1$^{r/r}$ CMV+. Stride length and

width were measured for four sets of hind paw prints. The rescue mice had restored gait patterns similar to wild type mice. Both Sepp1$^{r/r}$ CMV+ and wild type mice had significantly different stride length patterns compared to the ataxic irregular stride lengths of the Sepp1$^{-/-}$ mice. Two-way ANOVA determined there was a significant effect of genotype (**P= 0.0034) with Bonferroni's Post hoc analysis (*P<0.05) with values expressed as mean ± SEM of n= 6 per group (Fig. 6).

Restoration of spatial learning and memory. The Morris Water Maze (MWM) test was administered to assess whether spatial learning and memory were restored in the Sepp1$^{r/r}$ CMV+ mice. The visual platform test was given on the first day to introduce mice to learning to escape the water by climbing onto the platform, prior to beginning the training period. There were no significant differences between groups in the time (60 sec maximum) they took to find the visible platform (2-way ANOVA). During the training period, rate of learning was assessed by daily changes in escape latency times, which are measured as the time taken to climb onto the hidden platform during each trial (60 sec maximum). Sepp1 rescue mice learned at the same rate as the wild type group, as shown by lack of differences in escape times between the groups. Two-way repeated measures ANOVA (p>0.05) confirmed that there was no significant difference in escape latency during the 8 training days between Sepp1$^{r/r}$ CMV+ and Sepp1$^{+/+}$ mice (Fig. 7A). Escape latency times of both wild type and rescue mice were markedly less than those of Sepp1$^{-/-}$ mice.

During the probe trial, the time spent in each quadrant was recorded to assess if mice recalled where the platform was during the training period. There was no significant differences between genotype (Fig. 7B). The number of platform crossings was monitored during a 60 second swim. Swim speed and total distance traveled were recorded to control for factors other than learning and memory differences that could affect the results, such as motor coordination. We found no significant differences between wild type and rescue genotypes for time in each quadrant, number of platform crossings, swim speed or distance (two-way ANOVA, p>0.05) (Fig. 7C).

Discussion

This study sought to genetically restore Sepp1 gene expression in Sepp1 knockout mice as a proof of concept. We describe a strategy that takes advantage of the Cre-LoxP system, using the start codon in the LoxP site, to restore gene expression in the Sepp1 $^{-/-}$ mouse model. Previously the Schweizer lab generated a hepatically targeted Sepp1 transgene to rescue Sepp1$^{-/-}$ mice (158). The Schweizer lab generated a Sepp1 transgenic mice restricting SEPP1 to the liver by cloning the human SEPP1 cDNA, including the complete open reading frame and the two SECIS elements in the 3′-UTR, into exon 2 of the vector TTR exV3 and microinjecting the resulting minigene into mouse zygotes (259). However, our approach is novel in that we use the start codon in the existing LoxP site to genetically restore Sepp1 function, and can therefore rescue Sepp1 in any tissues with appropriate Cre expression and without additional transgenic manipulations.

Here we briefly review the Cre-LoxP system as it is pivotal to this study. The Sepp1$^{-/-}$ mice were constructed by utilization of the Cre-*LoxP* system by the Burk lab. Thus, this section provides essential background regarding this gene manipulating technology relevant to this study. In brief, the Cre-*LoxP* system, discovered in 1981, utilizes Cre recombinase protein that originates from the P1 bacteriophage (260). *LoxP* sites are part of the Cre-*LoxP* site-specific recombination system. *LoxP* sites consist of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region(261).

The fundamentals of the system are based on one Cre molecule that binds to the first and last 13 bp regions of a lox site forming a dimer or two Cre molecules binding at one *LoxP* site. This dimer then binds to a dimer on another lox site to form a tetramer (260). Lox sites are directional and the two sites joined by the tetramer are parallel in orientation. Recombination occurs in the asymmetric spacer region in which directionality of the recombination site is dependent on this 8bp region. The double stranded DNA is cut at both *LoxP* sites by the Cre protein. The strands are then rejoined with DNA ligase in a quick and efficient process. Two arrangements of the *LoxP* sites can be designed. The result of recombination depends on the orientation of the *LoxP* sites (261). For two lox sites on the same chromosome arm when two *LoxP* sites are in opposite orientation to each other, it will cause an inversion of the intervening DNA,

while a direct repeat of *LoxP* sites will cause a deletion event (260–262). Alternatively, if the two *LoxP* sites are oriented in the same direction as each other, this dictates an excision of the flanked DNA, leaving only one remaining *LoxP* site (262). If *LoxP* sites are on different chromosomes it is possible for translocation events to be catalyzed by Cre induced recombination (262). Two plasmids can be joined using the variant lox sites (261).

The site-specific excision of a particular piece of DNA can be used to eliminate or inactivate the endogenous gene or a transgene, or to activate a transgene (263). The Cre-LoxP system is typically used to generate conditional knockout animals. In some cases, researchers may choose to generate a conditional knockout due to embryonic lethality of a complete whole body knockout of a gene of interest. In other cases, researchers may use this method to study the function of a gene when it is absent or knocked out in a specific organ or cell population.

In this study, we demonstrate the novel application of the Cre-*LoxP* system to restore the SEPP1 gene in Sepp1$^{-/-}$ mice using the *LoxP* start codon. Further details regarding genetic restoration of the Sepp1 gene, using this unique application of the Cre-*Lox P* system, will be discussed in this study.

Our unique strategy of using the existing Cre-*LoxP* sites completely restores the SEPP1 gene. Sepp1$^{r/r}$ CMV+ mice have restored neuromotor function as observed in the vertical pole and stride tests. Furthermore, Sepp$^{r/r}$ CMV+ mice had normal spatial learning and memory and did not exhibit any of the deficits seen in Sepp1$^{-/-}$ mice when administered the Morris Water Maze test. Sepp$^{r/r}$ CMV+ mice had restored gait compared to Sepp$^{-/-}$ mice that have irregular stride length due to the dragging of the hind limbs as previously observed.

Sepp1$^{-/-}$ mice have been shown to have abnormal sperm morphology. Structural differences in sperm have been implicated as being central to the infertility of Sepp1$^{-/-}$ mice (10). Sepp1$^{r/r}$ CMV+ male mice were able to sire offspring, demonstrating that genetic rescue restored fertility, one of the most critical deficits seen in Sepp1$^{-/-}$. In a previous unpublished study by our group, we compared sperm morphology from cauda epididymides of Sepp1$^{r/r}$ CMV+ mice as well as those of Sepp1$^{+/+}$ and Sepp1$^{-/-}$ mice. Microscopy imaging observations confirmed that the rescue mice, Sepp1$^{r/r}$ CMV+, had

completely normal sperm morphology similar to Sepp1$^{+/+}$ absent of any kinks in the flagellum or narrowing at the posterior midpiece (Fig. 8). Sepp1 rescue mice had normal sperm motility patterns and sperm numbers as compared to the controls. Ultimately, the ability of Sepp1 rescue mice to sire pups indicates that infertility seen in male Sepp1$^{-/-}$ mice was negated, with neuromotor and learning and memory deficits recovered by this unique genetic rescue method.

We developed this unique strategy as a means to study the specific function of Sepp1 in the future. Several studies have investigated whether Sepp1 is the only source of plasma Sepp1 and Se distribution within the body, or whether Sepp1 may also have specific local functions in other organs (252). One study examined a liver-specific inactivation of Trsp, the gene for selenocysteine tRNA, which removes Sepp1 from plasma, causing a remarkable decrease in serum selenium levels and reducing kidney selenium to 36% of wild-type levels (252). However, the liver-specific Trsp knockout mice did not exhibit any neurological impairments and brain selenium levels remained unaffected, unlike Sepp1$^{-/-}$ mice. This indicates that hepatically derived Sepp1 is responsible for the transport function in plasma, while in brain, Sepp1 has a second, essential role that needs further investigation (252). This illuminating study provided evidence that the brain can maintain its Se and selenoenzyme levels in the absence of hepatic Sepp1 production, and therefore is largely independent of plasma Se levels as long as local Sepp1 expression in brain is preserved (252).

Another study generated a liver-specific rescue of human SEPP1 under control of a hepatocyte-specific transthyretin promoter in Sepp1$^{-/-}$ mice (259). This study sought to define the role of liver-derived circulating Sepp1 in contrast with locally expressed Sepp1. Secreted human Sepp1 was detectable in serum from SEPP1-transgenic mice (259). Selenium content and selenoenzyme activities in serum, kidney, testis and brain of Sepp(-/-;SEPP1) (SEPP1-transgenic Sepp(-/-)) mice were increased compared with Sepp$^{-/-}$ controls (259). Mice fed a selenium-adequate diet (0.16-0.2 mg/kg of body weight) showed that liver-specific expression of SEPP1 rescued the neurological defects of Sepp$^{-/-}$ mice and rendered Sepp$^{-/-}$ males fertile (259). This suggest that liver derived Sepp1 critically transports Sepp1 to male reproductive organs, most likely inutero, during an essential period for reproductive development. Additionally, despite

Sepp1 being synthesized locally in small amounts in testes (264), and providing distribution of other essential selenoproteins, for normal sperm development, Sepp1 expression in reproductive organs is dependent of liver derived Sepp1 transport. When fed on a low-selenium diet (0.06 mg/kg of body weight), Sepp(-/-;SEPP1) mice survived 4 weeks longer than $Sepp^{-/-}$ mice, but ultimately developed the neurodegenerative phenotype (259). These results confirm that plasma Sepp1 derived from hepatocytes is the main transport form of selenium supporting the kidney, testis and brain, which is particularly even more critical under a Se deficient diet (259). However, local Sepp expression appears to be required to maintain selenium content in selenium-privileged tissues such as brain and testis during dietary selenium restriction (259) .

Taken together, these studies provide evidence of an alternative role for Sepp1 distinct from its transport function. The potential of locally expressed Sepp1, not via plasma transport, having a specific function warrents further investigation in which this animal rescue model we established in this chapter provides the perfect tool.

In this segment of our study, we demonstrate that in generating this Sepp1 rescue model by this method, one can restore gene expression globally to all tissues by breeding Cre mice expressed in cells of your interest to a specifically designed knockout mouse, such that recombination would restore the genetic sequence in frame with its start codon. This unique application may allow researchers to study any gene of interest in highly specific cell populations. Researchers interested in such an approach to elucidate a specific function of a gene can specifically design the knockout mouse to have LoxP sites in which the LoxP start codon will remain in frame with the gene of interest following Cre recombination.

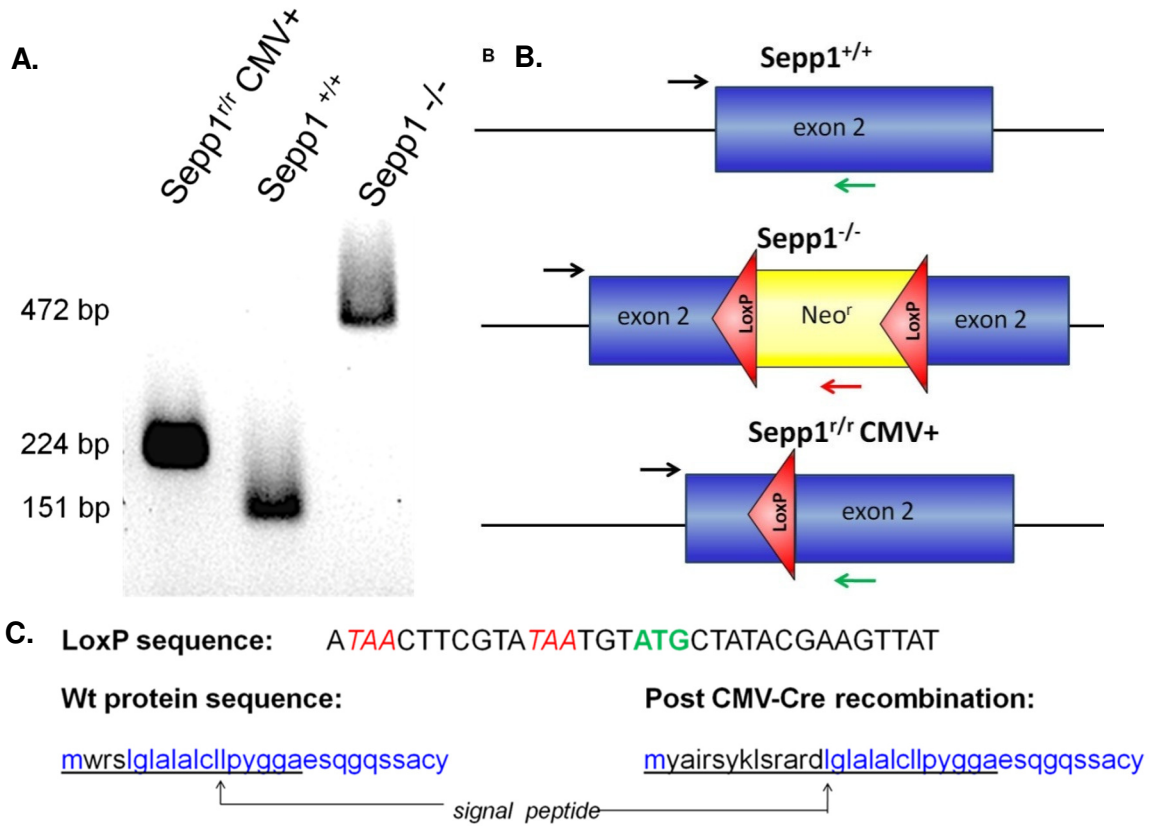


Figure 4. Generation of Sepp1$^{r/r}$ CMV+ rescue mice. (A) Polymerase chain reaction (PCR) genomic DNA was screened to confirm the genotypes of all animals. (B) Schematic of pre-and post-recombination of Sepp1$^{-/-}$ with Sepp1 CMV-Cre mice in which the Neo^{r} containing construct flanked by *lox P* was excised following breeding with CMV-Cre mice. Black, green and red arrows indicate primer locations. (C) Sequence comparison between Sepp1 wild type and recombined Sepp1$^{r/r}$ CMV+ showing the *lox P* site start codon remained in frame with the Sepp1 open reading frame to restore Sepp1 translation.

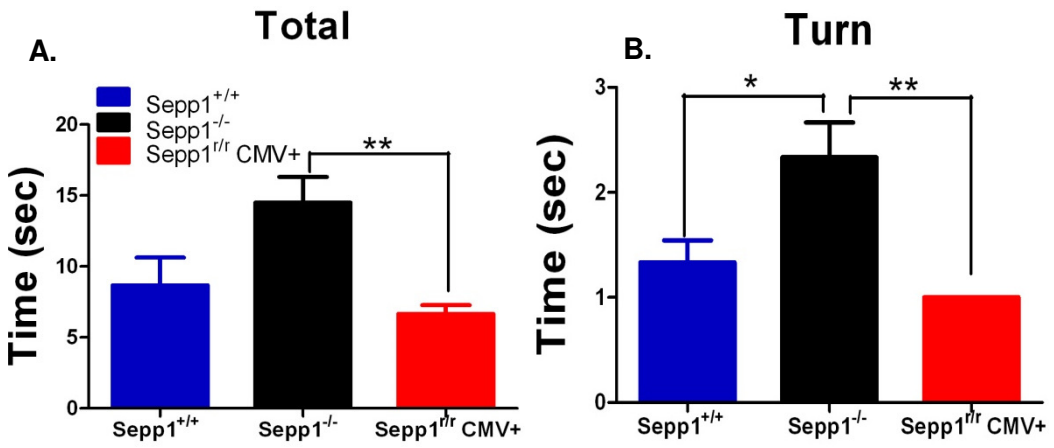


Figure 5. Sepp1$^{r/r}$ CMV+ mice have normal motor coordination. (A) Total time taken for Sepp1$^{r/r}$CMV+ rescue mice to descend the pole on the pole test was similar to WT and significantly faster than Sepp1$^{-/-}$ mice as reflected in one way ANOVA (P=0.0086) results. (B) Time taken for mice to coordinate and turn on the pole before descending was significantly less for the Sepp1 rescue mice than Sepp1$^{-/-}$ mice, and similar to normal WT, one way ANOVA (P=0.0024) with Bonferroni's Post hoc analysis performed for both (*P<0.05, **P<0.01). Values are expressed as mean ± SEM.

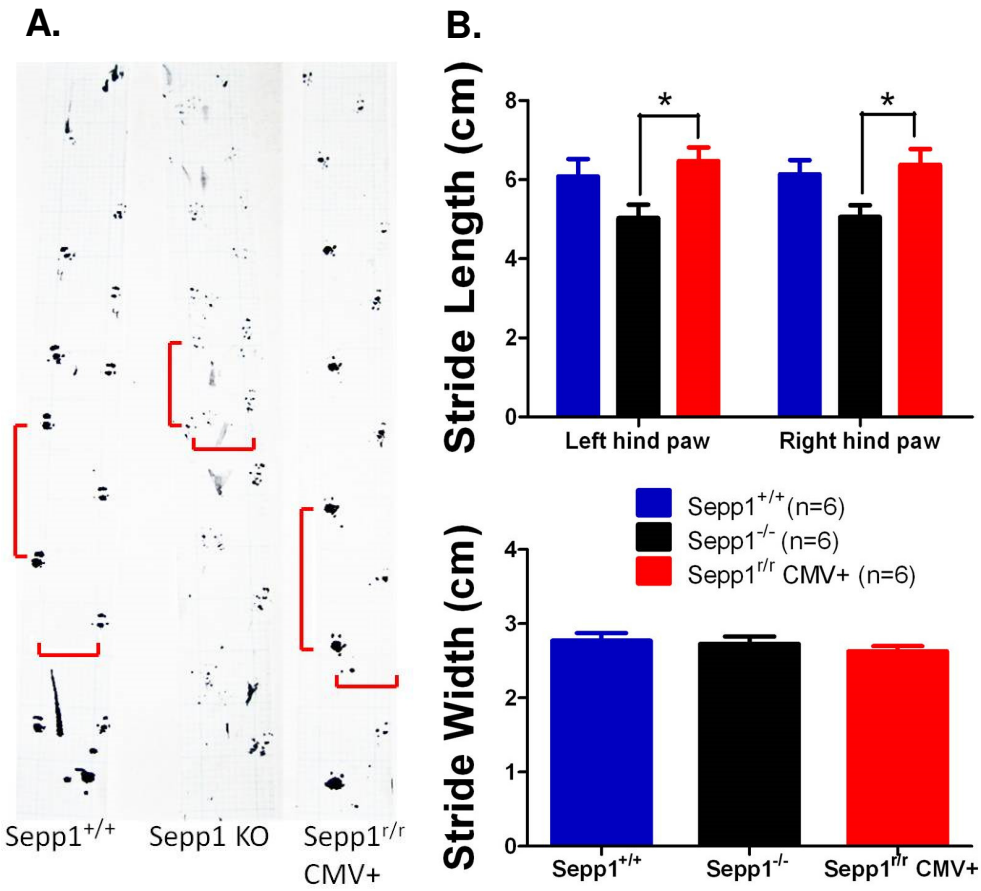
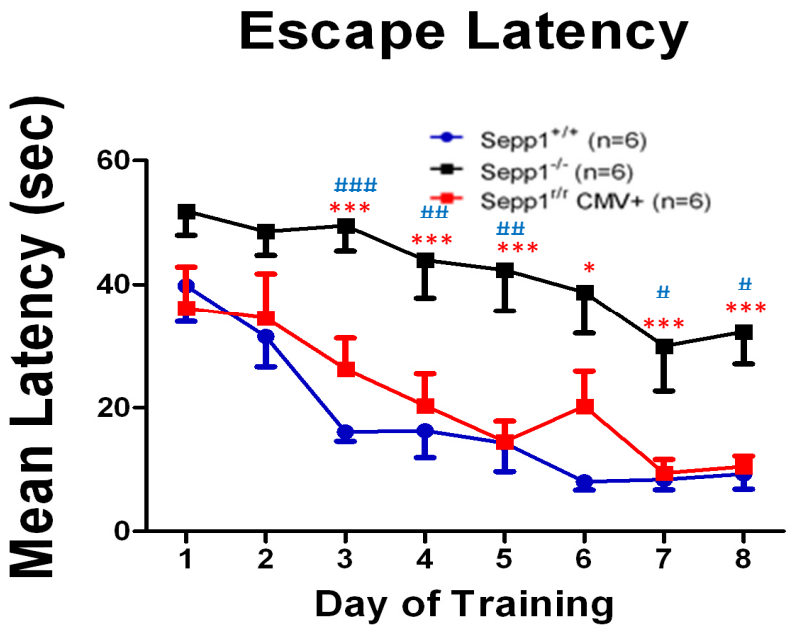
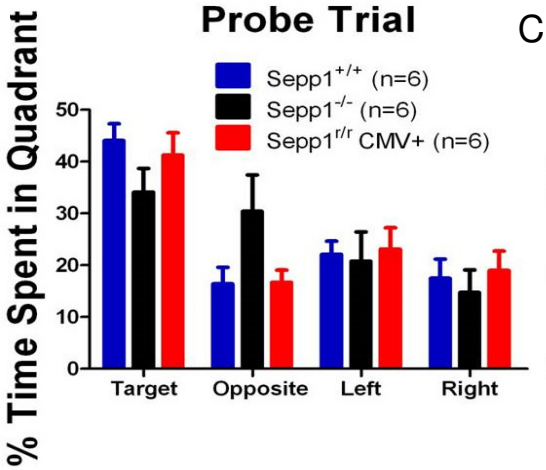


Figure 6. Abnormal gait exhibited by Sepp1$^{-/-}$ mice is restored in Sepp1 Rescue mice. (A) Representative paw print showing gait patterns of Sepp1$^{+/+}$, Sepp1$^{-/-}$, and Sepp1 rescue mice. (B) Analysis of stride lengths and widths. Two-way ANOVA of stride length (P=0.0034) with Bonferroni post hoc analysis showing the differences between genotype are significant (P<0.05) . Values are expressed as mean ± SEM of n= 6 per group. Length and width of hindpaw prints were measured from the center of each paw for each set of prints as indicated by the red lines.

A.



B.



C.

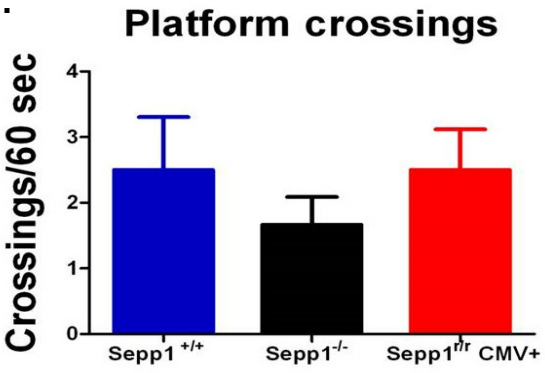


Figure 7. Sepp1$^{r/r}$ CMV+ mice have restored spatial learning and memory. Mean latency to escape via the platform between wild-type, Sepp1$^{r/r}$ CMV+mice, and Sepp1$^{-/-}$. Differences are statistically significant between Sepp1$^{-/-}$ compared to Sepp1$^{+/+}$ (*) and Sepp1$^{r/r}$ CMV+mice (#), respectively. (B-C) Percentage of time and number of virtual platform crossings during the probe trial determined if the mice recalled where the platform was. All results were analyzed by two-way repeated measures ANOVA followed by Bonferroni's post hoc analysis. Values are expressed as mean ± SEM. (ǂ) Sepp1$^{-/-}$ mice data were from a MWM experiment performed on a different day under the same parameters and are present only to show inherent deficiencies of this genotype for relative comparison to normal behavior.

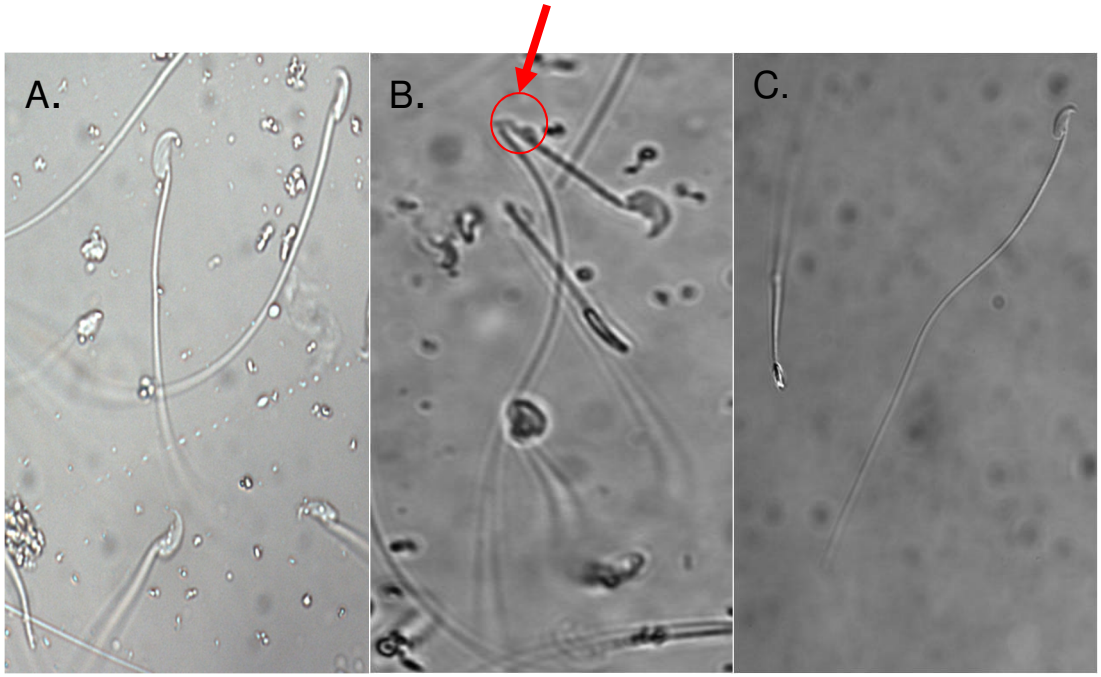


Figure 8. Sepp1$^{r/r}$ CMV+ (Rescue) mice have normal sperm morphology and motility. Representative images of (A) Sepp1$^{+/+}$ normal cauda epididymal sperm, (B) Sepp1$^{-/-}$ extended flagellum, sharp bend and narrowing (red arrow) at the posterior midpiece in cauda epididymal sperm. (C) Sepp1$^{r/r}$ CMV+ normal flagella and motility.

Chapter III

SEPP1 CONDITIONAL BRAIN RESCUE IN SEPP1$^{-/-}$ MICE RESTORES SYNAPTIC PLASTICITY

Abstract

Selenoprotein P (Sepp1) transports selenium throughout the body, particularly to the brain and testes. Selenoprotein P knockout (Sepp1$^{-/-}$) mice have severe neurological impairments that include motor function deficits, impaired hippocampal synaptic function and memory. Se supplementation alleviates some of these neurological deficits. However, synaptic plasticity is impaired in Se supplemented Sepp1$^{-/-}$ mice, as shown by deficits in long-term potentiation (LTP), a cellular model for learning and memory. We hypothesize that Sepp1 has a localized function in the brain besides transporting selenium. To determine the direct role for Sepp1 in the brain, expression of Sepp1 was restored only in forebrain neurons using a novel variation of the Cre-*LoxP* system. We assessed motor function and synaptic plasticity in 12-24 week old Sepp1$^{+/+}$ CaMKII-Cre (wild-type), Sepp1$^{-/-}$, and Sepp1$^{-/-}$CaMKII-Cre (Sepp1 Brain Rescue). Sepp1 Brain Rescue mice exhibited slightly impaired motor function compared to Sepp$^{+/+}$ mice, as shown by performance on the Rotarod and pole tests. The deficits in LTP previously reported in Sepp1$^{-/-}$ mice were restored, revealing a specific local function of Sepp1 in synaptic plasticity that is independent of its role in transporting Se from hepatocytes.

Introduction

Selenoprotein P (Sepp1) is important for synaptic plasticity and memory. In the brain, Sepp1 is primarily found associated with neurons and ependymal cells (91,265). Recent studies showed that the Sepp1$^{-/-}$ genotype or Se deficiency each lowered Se by similar amounts in cortex, midbrain, brainstem and cerebellum. However, in the hippocampus, Se was lowered by deletion of SEPP1 but not by Se deficiency, suggesting that Sepp1 is more important for maintaining Se in hippocampus than in other brain regions (156).

Mice with a deletion of the SEPP1 gene have severe neuromotor function deficits, abnormal gait, and deficits in hippocampal synaptic transmission, a region involved in memory formation(216). This results in disruption of spatial learning and a severe deficit in the long-term potentiation (LTP) memory model (216). These mice also exhibit spasticity and hyperreflexia that coincide with the deficient synaptic plasticity and widespread neurodegeneration. These mice also develop behavioral abnormalities such as impaired mobility and seizures, and die soon after weaning (103,151). These conditions can be exacerbated by dietary Se restriction and some abnormalities can be rescued by Se supplementation (113,162,266–268) (269). Furthermore, this neurological phenotype has been reported to be exacerbated in males compared to female mice lacking the SEPP1 gene (113).

Regardless of Se supplementation, Sepp1$^{-/-}$ mice still exhibit severe axonal degeneration (266,267) as well as impairments in synaptic plasticity and learning and memory (161). We hypothesized that Sepp1 has a direct and localized role in the brain besides its well-known function as a transporter of selenium. We applied the same method as described in Chapter II to conditionally rescue SEPP1, but here in only forebrain neurons, utilizing a brain-specific Cre-recombinase mouse for breeding. This mouse model allows us to study the function of localized Sepp1 expression in the brain and investigate whether Sepp1 expression, restricted to only forebrain neurons, could regulate synaptic plasticity independent of the role of Sepp1 in selenium transport.

Materials and Methods

Animals

Animals were maintained as described in Chapter II. All animals in this study were provided water supplemented with 10 µM Se ad libitum in addition to the standard lab diets containing adequate Se (~0.25 ppm). Mouse cohorts for this study were sex and age matched between 12-24 weeks old. All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Generation of Sepp1$^{-/-}$, Sepp1$^{-/-}$CaMKII-Cre (Sepp1 BrainRescue), and Sepp1$^{+/+}$CaMKII-Cre mice

Sepp1$^{-/-}$ mice were obtained from the laboratory of Dr. Raymond Burk at Vanderbilt University. Mutant mice were backcrossed to C57BL/6J for at least 10 generations before arriving in our lab and were bred with our C57BL/6J colony to ensure congenic strains (143). Since male Sepp1 mice are infertile (143), Sepp1$^{+/-}$ mice were used for breeding to obtain littermate Sepp1$^{-/-}$ and Sepp1$^{+/+}$ pups.

Sepp1 Brain Rescue mice were generated by first breeding mice with a Cre recombinase driven by the CaMKIIα promotor (B6.Cg-Tg(Camk2a-cre)T29-1Stl/J, (Jackson Laboratories) to Sepp1$^{+/-}$ mice. Sepp1$^{+/-}$CaMKII-Cre heterozygous mice were then bred to each other to produce Sepp1$^{-/-}$CaMKII-Cre (Sepp1 Brain Rescue) mice. This Cre mouse line has previously been extensively characterized, and reported to exhibit Cre expression almost exclusively in forebrain structures beginning approximately P16 (270,271). All breeders were provided water supplemented with 10µM Se (in the form of sodium selenite). Pups were given 10µM Se supplemented water following weaning at 3 weeks of age. All experiments utilized Sepp1$^{+/+}$/CaMKII-Cre (referred to as Sepp1 WT) female and male wild type and Sepp1$^{-/-}$ littermate mouse controls. Non-littermate female and male Sepp$^{-/-}$ mice added to the cohort were acquired by standard Sepp1 mice breeding described in Chapter II. Confirmation of the expected recombination of Sepp1 was carried out by polymerase chain reaction (PCR) of extracted genomic DNA from mouse tails using specific primers to amplify a 151-bp product in the targeted region present in the wild type allele (forward-ACCTCAGCAATGTGGAGAAGCC, reverse-TGCCCTCTGAGTTTAGCATTG), and 472-

bp and 224-bp products specific for the knockout allele and floxed gene, respectively (forward-ACCTCAGCAATGTGGAGAAGCC, reverse-GATGATCTGGACGAAGAGCATCA). Products were analyzed on 1.5% agarose gels, and SYBR® Safe DNA Gel Stain (InVitrogen) was detected by UV imaging to confirm genotype.

Animal Behavior

Age matched adult female and male Sepp1 WT, Sepp1 Brain Rescue, and Sepp1$^{-/-}$ mice 12 to 24 weeks of age were evaluated for neuromotor and neurobehavior effects using standard behavior paradigms that included Rotarod, in addition to the vertical pole test, and stride test previously described in Chapter II.

Rotarod

The Rotarod test evaluates motor coordination and is known to detect cerebellar dysfunction. Initial speed for the rod began at 4 rpm and increased to 40 rpm over a 5 min period. Latency to fall was recorded as the time at which the mouse fell off the rod. The mice were given four trials per day with an inter-trial interval of 1 hour for two consecutive days. The average latency for each day was calculated for each mouse and analyzed.

Immunofluorescence assay

An anti-Sepp1 custom antibody was used to evaluate Sepp1 protein expression. Sepp1$^{+/+}$, Sepp1$^{-/-}$, and Sepp1 Brain Rescue mice between 12-24 weeks old were anesthetized with 2,2,2-tribromoethanol. Brains were harvested and flash frozen in liquid nitrogen. Parasagittal sections 10 µm in thickness were cut using a cryostat (Leica CM 1950) and mounted on slides. Mounted sections were incubated in 4% PFA for 5 min at room temperature, washed with PBS-T and PBS, and blocked with 5% NGS, followed by overnight incubation with custom rabbit anti-Sepp1 (1:50 dilution) (Proteintech). Slides were gently rinsed three times with PBS. Alexafluor 488-conjugated anti rabbit (InVitrogen) secondary antibody was applied to samples and incubated for 1 hr at RT in a light protective container. Samples were then treated with

Autofluorescence Eliminator Reagent (Millipore). Coverslips were mounted over samples using Vectashield Mounting Media with DAPI (Vector Laboratories). Sections were imaged using an Olympus IX81 DSU confocal microscope. Images were taken at the same exposure and were adjusted uniformly for brightness and contrast in ImageJ (NIH).

Western Blot analysis

Mouse brains were harvested from animals euthanized by CO_2 according to IACUC guidelines. Sera was isolated from blood collected via tail vein or during brain tissue harvest for electrophysiology experiments with a syringe after decapitation. Sera was separated from blood collected in microcentrifuge tubes by centrifugation at 3000 x g for 30 min at RT. Tissues were flash-frozen using liquid nitrogen, crushed, and lysed with CelLytic MT (Sigma Aldrich Co) according to the manufacturer's instructions. Protein concentration was measured using a Nanodrop 1000 (Thermo Scientific). Samples were adjusted to equal concentration and mixed with Laemmli Sample Buffer (Bio-Rad Laboratories) containing 5% beta-mercaptoethanol. Samples were denatured at 95℃ for 10 minutes. Protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–20% gradient Tris-HCl Criterion Precast gel (Bio-Rad Laboratories) and transferred to Immobilon-FL polyvinylidene difluoride (PVDF) membranes (LiCor Biosciences). Membranes were blocked with Odyssey Blocking Buffer (LiCor Biosciences) for 1 hr at RT, then incubated with primary antibody diluted in 1:4 Blocking Buffer:PBS-T (PBS with 0.025% Tween-20) for 1 hr at room temperature (RT).

Sepp1 protein expression in serum of Sepp1 Brain Rescue and littermate control mice was measured with rabbit anti-Sepp1 antibody diluted 1:1000 (Proteintech). Sera was diluted by adding 1µl into Laemmli Sample Buffer (Bio-Rad Laboratories). Goat anti-GPx1 (R&D Systems) (1:1000) and rabbit anti-ApoER2 (1:1000) polyclonal antibodies were used to assess protein expression in brain tissue of age matched Sepp1 WT, Sepp1$^{-/-}$, and Sepp1 Brain Rescue mice. Following washes in PBS, primary antibodies were detected with LiCor near-infrared fluorescent secondary antibodies in 1:4 blocking solution in PBS for 45 min followed by washes as described above. Mouse

polyclonal anti-tubulin, diluted 1:5000 (Sigma Aldrich Co.), was probed and used as a loading control. For sera protein loading control detection on the PVDF membrane, SimplyBlue Safestain (InVitrogen) was used according to manufacturer instructions. Protein was detected using the Odyssey® Infrared Imaging System (LiCor Biosciences). Scanning and analysis were performed with LiCor Odyssey Image Studio 2.1 software.

Hippocampus Slice Preparation and Field Potential Recordings

Sepp1 Brain Rescue mice and control littermates used for electrophysiology were given water supplemented with 10 µM Se ad libitum. Acute hippocampal slice preparation and electrophysiology were performed on mice 12 to 24 weeks of age using standard methods (272,273). Animals were deeply anesthetized with 330 mg/kg tribromoethanol. Brains were rapidly harvested and quickly placed in ice cold artificial cerebrospinal fluid (ACSF: containing in mM: 130.0 NaCl, 3.5 KCl, 24.0 $NaHCO_3$, 1.25 NaH_2PO_4, 1.5 $MgSO_4$, 2.0 $CaCl_2$) oxygenated with carbogen (95% O_2, 5% CO_2). Transverse brain slices, 350 µm in thickness, were cut using a vibratome (Leica VT 1000 S). Hippocampi were isolated from the slices and transferred to a nylon mesh holding chamber and allowed to equilibrate at room temperature for 30 min then at 32°C in oxygenated ACSF for another 30 minutes. All slices were permitted a minimum 1 hr recovery time before transfer to a recording chamber. During fEPSP recordings, slices were superfused with oxygenated ACSF at 32°C at 1-3 ml/min.

Extracellular field recordings were obtained from the stratum radiatum of the CA1 area. Field EPSPs (fEPSPs) were evoked by stimulation to the Schaffer collaterals and recorded with a glass microelectrode filled with 3M NaCl (resistance 1–5 MΩ). Prior to LTP induction, the input/output (IO) relationship of the slope of the CA1 fEPSP in response to 0-15 V stimuli (1 V increments) to the Schaffer collateral fibers was recorded. IO is a measure of synaptic strength. Input-output curves were obtained by varying stimuli from 0 to 15 V with 1 V increments. Paired-pulse facilitation was achieved with pairs of stimuli at 3V with interstimulus intervals of 10, 50, 90, 130, 170, 210 and 250 msec, with 30 sec between each pair of stimuli.

Long-term potentiation (LTP) was induced with high-frequency stimulation (HFS) following at least 20 min of stable baseline recording. HFS consisted of two trains of 100

Hz frequency stimulation and 1 sec duration with each train separated by a 20 sec interval. 3V stimulation was used to produce fEPSPs that were approximately 10-30% of maximum responses. Potentiation was measured as the normalized increase of the mean pEPSP following HFS normalized to the mean pEPSP for the duration of the baseline recording.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software. Sample size were determined through power analysis (257,258). Interaction between genotypes and sex was ascertained by Student's *t*-test and two-way analysis of variance (ANOVA) with Bonferroni's posthoc test for multiple comparisons. To determine genotype differences between experimental groups in the water maze training and quadrant entries in the probe trial, repeated-measures of ANOVA with Bonferroni's posthoc test were used. Data represent the mean ± SEM. Statistical significance was defined as $p<0.05$ for all statistical tests (Chapter II).

Results

Restoration of Sepp1 protein hippocampal expression and absence in Sepp1 Brain Rescue mouse sera are indicative of proper recombination of Sepp1 in forebrain neurons. Applying the novel technique developed and described in Chapter II but restricted to forebrain using the CaMKII promoter, Sepp1 Brain Rescue mice were generated to investigate the localized function of Sepp1 independent of Se transport from Sepp1 synthesized in hepatic cells. Verification of forebrain specific restoration of SEPP1 was assessed by standard genotyping as well as protein analysis. Sepp1 comprises approximately 60% of plasma selenium. Therefore protein analysis was performed on sera separated from blood on SDS-PAGE, followed by western blotting for Sepp1 protein. Sepp1 Brain Rescue sera was negative for Sepp1 protein due to absence SEPP1 gene expression, thus secretion of liver derived Sepp1 into the plasma (Fig.9).

Imaging analysis focused primarily on the hippocampus, the brain region for consolidating memory and learning, in which the synaptic physiology experiments in this study were conducted. Immunofluorescence labeling demonstrated that Sepp1 Brain Rescue mice brains expressed Sepp1 mainly in forebrain neurons with expression colocalized with DAPI labeling for nuclei (Fig. 10).

We assessed whether this conditional rescue of forebrain localized Sepp1 disturbed selenoprotein homeostasis. GPx1 is a ubiquitously expressed member of the Gpx-family suggested to have a role for redox-balance in modulating neuronal protection(274). Protein expression of GPx1 was assessed in hippocampal tissue of Sepp1 Brain Rescue, Sepp1 WT, and Sepp1$^{-/-}$ mice by Western Blot. The differences between genotypes were not statistically significant. However, GPx1 expression was decreased in Sepp1 Brain Rescue mice compared to that of Sepp1$^{+/+}$, while Sepp1 $^{-/-}$ mice was slightly greater relative to Sepp1 WT mice. These differences were not statistically significant (one-way ANOVA with Bonferroni Post-hoc test analysis) (Fig. 11).

ApoER2, a known receptor of Sepp1 in the brain, is normally downregulated upon ligand binding (275). We analyzed ApoER2 expression to assess whether the conditional genetic rescue of Sepp1 in the forebrain led to potential overexpression of

Sepp1. Western blot results show that Sepp1 Brain Rescue hippocampi had similar ApoER2 expression to that of Sepp1 WT, whereas ApoER2 was upregulated in Sepp1$^{-/-}$ due to absence of Sepp1 receptor binding but differences were not statistically significant (one-way ANOVA). (Fig. 11).

Neuromotor function and gait impairments are not completely recovered in Sepp1 Brain Rescue. Sepp1$^{-/-}$ mice have severe motor impairments encompassing irregular gait patterns described to include dragging of the limbs and uneven strides as well as motor coordination deficits (159,161). Sepp1$^{-/-}$ mice on Se deficient diet exhibit widespread neurodegeneration including dystrophic and degenerated axons in the pons and spinal cord (266). Gender specific neurobehavioral deficits have been reported in Sepp1-deficient mice raised on a standard lab diet (113). Raman et al. demonstrated that spontaneous locomotor activity, was greatly decreased in the male Sepp1 Se deficient mice while being slightly decreased in the female knockout animals, when compared to wild-type mice (113).

We utilized the vertical pole, rotorod, and stride tests to assess if SEPP1 gene expression restricted to only forebrain neurons was sufficient to recover some of the neuromotor behavioral deficits reported in Sepp$^{-/-}$ mice. The vertical pole test demonstrated that the ability of Sepp1 Brain Rescue mice to coordinate the turn before descending the pole was similar to Sepp1 WT. Time taken for Sepp1 Brain Rescue mice to coordinate and turn on the pole before descending was similar to normal Sepp1 WT mice in both females. However, when comparing between sex, Sepp Brain Rescue males performed slightly worse than Sepp1 Brain Rescue females (one-way ANOVA, $p>0.05$). Sepp1 male $^{-/-}$ mice performed significantly worse than Sepp1 WT controls (one-way ANOVA with Bonferroni's Post hoc analysis $*p<0.05$), (Fig. 13B). Total time taken for Sepp1 Brain Rescue mice to descend the pole was similar to Sepp1 WT in both females and males although Sepp1 Brain Rescue show slightly slower total descent time than Sepp1 WT controls, but faster than Sepp1$^{-/-}$ mice. Sepp1$^{-/-}$ males performed significantly worse than Sepp1 WT male controls ($*p<0.05$), but only slightly worse than Sepp1$^{-/-}$ female counterparts. When we analyzed by genotype regardless of sex, it showed that Sepp1$^{-/-}$ mice took longer to descend the pole compared to Sepp1

WT controls (one-way ANOVA with Bonferroni's Post hoc analysis,*$p<0.05$) (Fig. 13D). No with other statistically significant differences observed between other genotypes or for turn time. We subjected the mice to a stride test to determine whether the ataxia seen in Sepp1$^{-/-}$ mice was recovered in the Sepp1 Brain Rescue mice. No differences were seen between the groups determined by two-way ANOVA with Bonferroni's Post hoc analysis ($p>0.05$) (Fig. 13).

The Rotarod assay was used to evaluate balance, grip strength, and motor coordination. Our data show that Sepp1 Brain Rescue mice exhibit marginally impaired balance and motor coordination. Rotarod performance on Day 1 showed Sepp1 Brain Rescue latency to fall was significantly less than that of Sepp1 WT (1-way ANOVA, $p< 0.05$). Sepp1 Brain Rescue mice demonstrated improved motor coordination and balance on Day 2 as compared to Day 1. Sepp1 Brain Rescue mice improved and remained on the rod longer relative to the Sepp1$^{-/-}$ mice, but still did not perform as well as Sepp1 WT mice. Sepp1$^{-/-}$ mice performance on Day 2 was significantly less than that of Sepp1 WT (one-way ANOVA with Tukey's multiple comparison post hoc test, $p<0.05$) (Fig. 12). All groups showed improved latency on Day 2 (one-way ANOVA Tukey's multiple comparison post hoc test $p<0.0001$). Male compared to female mice in all groups appeared to perform more poorly, exhibiting a trend towards lower latency to remain on the rod. However, no statistically significant differences were observed when data were analyzed to compare performance between sex, within and among genotypes (2-way ANOVA, data not shown).

We subjected the mice to a stride test to determine whether the ataxia seen in Sepp1$^{-/-}$ mice was recovered in the Sepp1 Brain Rescue. Stride length and width were measured for four sets of hind paw prints. The Sepp1 Brain Rescue had similar gait patterns similar to wild type mice. Both Sepp1 Brain Rescue and wild type mice had similar stride length patterns to each other. Sepp1$^{-/-}$ mice also exhibited normal stride length thus there were no significant difference in stride length between genotypes Two-way ANOVA with Bonferroni's Post hoc analysis ($P>0.05$) (Fig. 14). Selenium supplementation in this cohort may have alleviated abnormal gait exhibited by Sepp1$^{-/-}$ mice. There were no differences in stride width as observed before in previous experiments (Chapter 2).

Synaptic plasticity is restored in Sepp1 Brain Rescue

To determine whether synaptic plasticity deficits observed in Sepp1$^{-/-}$ mice are due to absence of Se transport from the liver or attributed to the local action of Sepp1 in the brain, we studied basal synaptic transmission and synaptic plasticity in hippocampi of our conditional forebrain rescue mouse model. We examined the synaptic physiology in area CA1 of the hippocampus, a brain region crucial for normal spatial learning and memory formation. To establish the basal synaptic strength of each genotype we assessed the input-output (IO) of synaptic responses in the Schaffer collateral-CA1 synaptic. The presynaptic fibers were stimulated with increasing stimulus intensities and recording the evoked CA1 field excitatory postsynaptic potentials (fEPSPs) in stratum radiatum at Schaffer collateral synapses. As stimulus intensity increased, Sepp Brain Rescue mice exhibited a non-significant overall increase in synaptic efficacy compared to Sepp1 WT and Sepp$^{-/-}$ ($p>0.05$ two-way ANOVA) (Fig. 15A).

Paired-pulse facilitation (PPF) is a form of short-term plasticity. When two pulses at a short interpulse interval are given to the afferent pathway, the postsynaptic response to the second stimulus is increased when compared with the first response (162). PPF can provide information regarding the loci of the LTP expression, whether changes in the response is mediated presynaptically and most likely not postsynaptically. When LTP expression occurs at a presynaptic loci, then it may alter PPF potentiation. A smaller initial PPF is associated with an increase in PPF while a larger initial PPF was associated with a decrease in PPF with LTP (222). Alterations in PPF response within the same time course as LTP also suggest it is input specific and not due to nonspecific effects of high-frequency stimulation, changes in inhibition, active postsynaptic currents or their nonlinear summation, and PPF changed with the same time course as LTP (222). Sepp1 Brain Rescue mice exhibited PPF responses similar to that of Sepp1 WT mice. We observed no difference in paired pulse facilitation (PPF) in all groups measuring interpulse interval stimulus response over time ($p>0.05$ two-way ANOVA) (Fig. 15B).

LTP is a mechanism necessary to store a long-term memory, in which high frequency stimulation of neural pathway induces signal transmission that results in long-

lasting increased synaptic efficacy between neurons (214). LTP is characterized first by a transient and highly robust post-tetanic increase in the slope of the fEPSP that is followed by a sustained and less robust increase (216). We induced LTP in hippocampi of all mice genotypes by applying high-frequency stimulation (HFS) of two 100 Hz trains, 20 sec apart to assess a type of long-term plasticity exhibited in the Schaffer collateral-CA1 pathway important in memory formation. Sepp1 Brain Rescue male mice exhibited restored normal LTP similar to that of Sepp1 WT mice. Whereas, as previously published, Sepp1$^{-/-}$ mice exhibit abolished LTP (162) (Fig. 16). Previous data demonstrated potential sex differences in synaptic plasticity of Sepp1$^{-/-}$ mice in regards to LTD (Bellinger et al., unpublished). When we conducted sex comparisons within and between genotypes we observed no differences (data not shown).

Discussion

Sepp1 is known predominantly to be synthesized in the liver for selenium synthesis and distribution (Fig.1). Sepp1 has also been shown to be synthesized in small amounts in neurons and oligodendroglial cells of the brain (276). Sepp1 plays a vital role in the homeostasis of Se in the brain (103). All regions of the brain are dependent on Sepp1 for selenium (277). Deletion of Sepp1 under Se deficient conditions results in neurological impairment with ataxia and seizures, which are well documented in many published studies (101,161). Deletion of Sepp1 in mice have also been shown to result in hippocampal-dependent long-term potentiation deficits, the cellular basis for learning and memory. Previous studies have demonstrated that alterations in synaptic transmission and LTP in Sepp1 are irreversible despite Se supplemented diets (162).

We show that Sepp1 has a localized function in the brain regulating synaptic plasticity, independent of its known role of Se transport. We demonstrate local Sepp1 brain function to generate a conditional forebrain rescue expressing Sepp1 solely in forebrain neurons in otherwise Sepp1$^{-/-}$ mice. Previous studies report that Sepp$^{-/-}$ mice have synaptic plasticity impairments in which LTP is abolished. We show that the Sepp1 Brain Rescue mice have restored synaptic plasticity as evidenced by restored LTP. To confirm restoration of SEPP1 to forebrain neurons, we analyzed protein from sera, immunohistochemistry of Sepp1 Brain Rescue hippocampi, and expression of other

selenoproteins in the brain. We confirmed that Sepp1 Brain Rescue mice sera protein expression is absent in the body, as seen in Sepp1$^{-/-}$, compared to Sepp1$^{+/+}$ mice, through Western blot protein analysis (Fig. 9). This shows SEPP1 is not expressed in other parts and not being transported and synthesized from liver as it is absent from the sera. Further imaging analysis focusing on the hippocampus show that Sepp1 Brain Rescue mice brains expressed Sepp1 mainly in forebrain neurons with expression colocalized with DAPI labeling for nuclei (Fig. 10). Based on CA1 area landmarks, immunostaining outside of the cell bodies around the DAPI staining is largely dendritic. These results indicates that Sepp1 Brain Rescue mice have restored Sepp1 protein expression in forebrain neurons of hippocampal CA1 area, similar to Sepp1 WT mice, whereas Sepp1 signal is absent in Sepp1$^{-/-}$ (Fig.10).

We assessed whether this conditional rescue of forebrain localized Sepp1 disturbed selenoprotein homeostasis. GPx1 is a ubiquitously expressed member of the Gpx-family suggested to have a role for redox-balance in modulating neuronal protection (274). GPx1 protein expression was decreased in Sepp1 Brain Rescue mice compared to that of Sepp1$^{+/+}$, while in Sepp1$^{-/-}$ mice it was slightly greater relative to Sepp1 WT mice, suggesting a potential decrease in oxidative stress in the Sepp1 Brain Rescue mice. These differences were not statistically significant (one-way ANOVA with Bonferroni Post-hoc test analysis).

Sepp1$^{-/-}$ mice have severe motor impairments encompassing irregular gait patterns described to include dragging of the limbs and uneven strides as well as motor coordination deficits (159,161). Sepp1$^{-/-}$ mice on Se deficient diet exhibit widespread neurodegeneration including dystrophic and degenerated axons in the pons and spinal cord (266). Gender specific neurobehavioral deficits have been reported in Sepp1-deficient mice raised on a standard lab diet (113). Raman et al. demonstrated that spontaneous locomotor activity, was greatly decreased in the male Sepp1-deficient mice while being slightly decreased in the female knockout animals, when compared to wild-type mice (113).

We utilized the vertical pole, rotorod, and stride tests to assess if SEPP1 gene expression restricted to forebrain neurons was sufficient to recover some of the neuromotor behavioral deficits reported in Sepp$^{-/-}$ mice. In the vertical pole test, which

involves the basal ganglia and cerebellum, upon coordination of the turn, when comparing between sex, Sepp Brain Rescue males performed slightly worse than Sepp1 Brain Rescue females (one-way ANOVA, $p>0.05$) (Fig. 13A). This is not surprising since previous reports have shown that Sepp1$^{-/-}$ males exhibit more severe phenotypes even with Se supplementation (113). As a characteristic of abnormal gait, Sepp1$^{-/-}$ mice tend to drag their hind limbs thus hindering the ability of mice to descend the pole. Raman et al., reported that Sepp$^{-/-}$ male mice performed significantly worse than Sepp$^{-/-}$ females during the inversion on the pole test even after Se supplementation (113). In total time taken to descend, Sepp1$^{-/-}$ males performed significantly worse than Sepp1 WT male controls ($*p<0.05$) (Fig. 13B), but only slightly worse than Sepp1$^{-/-}$ female counterparts, similar to the results in the Ramen et al. study regarding Sepp$^{-/-}$ mice.

We subjected the mice to a stride test to determine whether the abnormal gait or ataxia typically observed in Sepp1$^{-/-}$ mice was recovered in the Sepp1 Brain Rescue (Fig. 14). Even though in previous experiments (Chapter 2), Sepp1$^{-/-}$ mice exhibited ataxic, irregular stride lengths, Sepp1$^{-/-}$ mice in these experiments have improved performance, potentially due the Se supplementation that was provided for this group beginning at 3 weeks of age postnatal (113). Stride width, usually observed in ataxia, were not impaired in Sepp1$^{-/-}$ mice on lower Se diet, as previously reported (Chapter II). The absence of any stride width impairments in Sepp1$^{-/-}$ mice in the stride test regardless of an adequet or high Se supplemened diet, suggest that deletion of Sepp1 may not critically effect cerebellum function, which ataxic gait is associated, relative to other brain regions such as the hippocampus,a region important for learning and memory, where absence of Sepp1 results in more pronounced effects. Therefore lending further support to our Sepp1 Brain Rescue model in arguing for a specific role of Sepp1 in the forebrain.

The Rotarod assay was used to evaluate balance, grip strength, and motor coordination. Our data show that Sepp1 Brain Rescue mice exhibit marginally impaired balance and motor coordination (Fig. 12). No statistically significant sex-specific differences were observed. Overall, the results of the neurobehavior assessments show that forebrain-restricted Sepp1 rescued some of the neuromotor deficits such as

irregular gait, descending the pole, motor coordination learning. However Sepp1 Brain Rescue, despite mostly not statistically significant, showed that not all motor function was completely recovered as the forebrain does not completely control all motor function. These result provides further evidence that Sepp1 is not expressed in other parts of the brain, such as the cerebellum (part of the hind brain), which helps control balance, posture, coordination of voluntary movements, motor learning, and cognitive behavior.

We examined the synaptic physiology in area CA1 of the hippocampus, a brain region crucial for normal spatial learning and memory formation. Measuring basal synaptic transmission helps determine the normal synaptic strength of the specific genotype and brain health prior to any artificial stimulation. The basal synaptic strength in Sepp Brain Rescue mice exhibited normal synaptic efficacy similar to Sepp1 WT and $Sepp^{-/-}$. PPF helps determine if alterations in LTP are presynaptic or postsynaptic actions. When LTP expression occurs at a presynaptic loci, then it may alter PPF potentiation (222). Sepp1 Brain Rescue mice in exhibited PPF responses similar to that of Sepp1 WT mice. We observed no difference in paired pulse facilitation (PPF) in all groups, arguing against any presynaptic changes. LTP, a form of synaptic plasticity, in the CA1 region of the hippocampus has been the primary model by which to study the cellular and molecular basis of memory (213). LTP Sepp1 Brain Rescue male mice exhibited restored normal LTP similar to that of Sepp1 WT mice. Whereas, despite selenium supplementation greater than what is considered adequate levels, $Sepp1^{-/-}$ mice exhibit abolished LTP, which has been shown to correlate with learning and memory deficits as determined by behavioral tests (216). Our data demonstrate that the conditional rescue of Sepp1 expression restricted to forebrain neurons in $Sepp1^{-/-}$ mice is sufficient to recover severe LTP deficits. This supports our hypothesis that neuronal expression of Sepp1 in hippocampal cells is necessary for LTP, independent of its transport function.

$Sepp1^{-/-}$ mice have decreased brain Se comparable to that of wild-type animals raised on a 0 Se diet (102,103,161). $Sepp1^{-/-}$ mice have a similar decrease of Se in the cortex, midbrain, brain stem, and cerebellum, while Se in the hippocampus is decreased by Sepp1 gene deletion but not by 0 Se diet (160,278). $Sepp1^{-/-}$ mice on minimal

adequate Se supplementation of 0.1mg/kg have been previously reported to have synaptic plasticity deficits correlated with spatial learning impairments (216). Sepp1$^{-/-}$ mice on less than 0.1mg/kg exhibit high mortality rates (161). In a seminal study, Peters et al. demonstrated that Sepp1$^{-/-}$ mice have an increase in synaptic transmission but lack changes in the fiber volley amplitude, suggesting that Sepp1 deficiency contributes to defects in postsynaptic function (162). However, it was later determined that after several more generations of mice the increased synaptic transmission were no longer observed in Sepp1$^{-/-}$ mice. We did not find any alteration in mice in either synaptic transmission or fiber volley amplitude in Sepp1$^{-/-}$. However, we did observe a slight increase in synaptic strength in the Sepp1 Brain Rescue but it was not statistically significant. Although not significant, the enhanced basal synaptic transmission may suggests that synapses in Sepp1 Brain Rescue mice might have a slightly improved ability to undergo synaptic plasticity (162).

Sepp1$^{-/-}$ mice also show a decrease in paired pulse facilitation PPF at 20, 40 and 120 ms inter-pulse intervals, which is distinctive of presynaptic dysfunction (162). This phenomenon can be attributed to residual calcium in the presynaptic terminal that facilitates neurotransmitter release upon the second stimulation resulting in the subsequent increase in the post synaptic response [26] (162,221). Results from Peters et al. suggest that deletion of Sepp1 alters both presynaptic (altered neurotransmitter release) and postsynaptic properties (enhancement of fEPSP slopes) (162).

In addition, Sepp1$^{+/+}$ mice on a selenium-deficient diet of 0 mg/kg were shown to have altered synaptic strength and decreased LTP similar to that of Sepp1$^{-/-}$ mice on 1 mg/kg Se diet (162). Importantly Peters et al. also reported Sepp1$^{-/-}$ mice, despite Se supplementation (1mg/kg Se diet) , exhibited severe long-term potentiation (LTP) deficit, the cellular underpinnings of learning and memory, and is a predominantly post-synaptic dependent activity (162).

Targeted inactivation of Sepp1 by way of deletion of the gene encoding tRNA[Ser]Sec biosynthesis of all hepatocytic selenoproteins, resulted in reduced serum and kidney Se levels comparable to that of Sepp1 KO mice, yet neurological dysfunction was not observed (158,279,280). This suggests that neurological function may not be dependent on liver derived Sepp1 synthesis, and therefore implicating a

potential local role of Sepp1 in the brain. The same group who performed the study on mice with Sepp1 targeted inactivation, followed up with a model using a liver-specific genetic expression of Sepp1 in Sepp1 KO mice on Se adequate diets (252). This study showed that the liver-specific Sepp1 rescue mice restored the neuromotor deficits observed in Sepp1 KO mice on the Rotarod test, confirming that Sepp1 is the primary transport form of Se supporting the kidney, testes, and brain (158). However, it did not show if this liver-specific Sepp1 expression mouse model resulted in recovery of the other neurological deficits such as synaptic plasticity, learning and memory. They conclude that local Sepp1 expression is required to maintain selenium content in selenium-privileged tissues such as brain and testis during dietary selenium restriction despite their findings confirming the necessity of liver-derived Se transport (158).

To understand when Sepp1 was expressed during the development of the Sepp1 Brain Rescue mice, we briefly discuss details of the Cre recombinase utilized to generate the rescue of the SEPP1 gene. The CaMKIIα promoter in these Cre expressing mice has been demonstrated to have targeted specificity to the hippocampus and necocortical tissues that have central roles in learning and memory (270). Originally intended for use for conditional knock-outs, the CaMKIIα-Cre promoter lacks activity during prenatal and perinatal periods, reducing the potential of developmental defects due to a gene knockout (270). In our current study, this is advantageous as the Sepp1 Brain Rescue mice essentially have similar development during the prenatal and perinatal periods as Sepp1 KO mice.

CA1 pyramidal cells undergo neurogenesis between E10 and E18 (270,281) and enter the postmitotic state by P0 and are well differentiated by P7, with fully established synaptic connections (282–284). Recombination did not significantly alter the organization of the various brain nuclei or in the arrangement of cell layers within major brain nuclei and no overt behavioral or morphological abnormalities in several *Cre* transgenic lines (270). CaMKII mRNA was also shown to be in both the hippocampus and cortex by Northern blot, with expression similar to that in hippocampus (270). However, immunohistology showed dense staining in the CA1 region pyramidal cells of the hippocampus and somewhat lighter staining in the CA3 region than in the rest of the hippocampus and cortex (270). One explanation provided by the authors was that the

*CaMKII*α promoter is particularly active in CA1 pyramidal cells, therefore recombination is more efficient and is reached more frequently in these cells as opposed to other forebrain cells (270). Another possibility was that CA1 pyramidal cells provide environmental factors that are favorable for the synthesis, stability, transport, or activity of the Cre recombinase (270). We confirm in our study through immunohistochemistry analysis, that relative Sepp1 expression is restored in the CA1 region of Sepp Brain Rescue mice hippocampus similar to that of $Sepp^{+/+}$ and is absent in $Sepp^{-/-}$ (Fig. 10).

Transcription of mRNA of *CaMKII* driven by the *CaMKII*α promoter has been shown to be silent until several days after birth (270,285). Cre recombination was reported to begin at P19 which coincides with the middle or end of the third postnatal week in the CA1 pyramidal cells, indicating that this type of recombination is not inhibited by the highly differentiated, post-mitotic state of the CA1 neurons (270). As the mice aged, recombination spread to more cells but was restricted to the CA1 pyramidal cell layer (270). By P29, the adult recombination pattern was established and remained restricted to the CA1 area (270).

Studies have shown that Sepp1 is more important for maintaining selenium in the hippocampus than in other brain region and prioritizes the brain in the selenium hierarchy (277) thus supporting our hypothesis that localized Sepp1 in the brain has an independent neurological function. Studies of mice with $Sepp1^{-/-}$ mice shows that brain and testis selenium levels are largely dependent on Sepp1 (101). Brain and testis maintained their selenium better than did liver, kidney, and muscle when dietary selenium was limiting but testis selenium fell sharply in the group fed the deficient diet (277). Also, Sepp1 selenium uptake between brain and testis has been shown to be different. Deletion of Sepp1 and selenium deficiency in $Sepp1^{+/+}$ each lowered selenium a similar amount in cortex, midbrain, brainstem, and cerebellum. Selenium in the hippocampus was lowered by deletion of Sepp1 but not by selenium deficiency (277).

Valentine et al., showed that the LTP deficit in $Sepp1^{-/-}$ mice is independent of the amount of HFS presynaptic input, but does not rule out the possibility of occluded LTP (216). The study demonstrated that there was no stimulation threshold preventing LTP induction and therefore HFS did not induce potentiation greater than the standard two train 100 Hz protocol. The results were concluded after administering $Sepp1^{-/-}$ slices

were given multiple HFS protocols (2 trains of 100 Hz stimulation, separated by 20 sec repeated 4 times with each set given 5 minutes apart).

Importantly, selenium depletion through dietary restriction results in the absence of selenocysteine and subsequent early translation termination at the site of selenocysteine incorporation of all the selenoproteins, including Sepp1. Therefore, *Sepp1*$^{+/+}$ (0 Se diet) have reduced selenium in the CNS coupled with a decrease of Sepp1(162). Similar to the *Sepp1*$^{-/-}$, LTP also is altered in hippocampal slices obtained from Sepp1$^{+/+}$ mice on 0 Se. Similar to the Sepp1$^{-/-}$ slices, Sepp1$^{+/+}$ (0 Se diet) slices do not exhibit LTP in response to HFS compared to the *Sepp1*$^{+/+}$ mice fed 1 mg Se/kg (216). Brain function is severely compromised in mice expressing *C-terminal deleted Sepp1* mice fed a selenium-deficient diet but not as severely as in Sepp1$^{-/-}$ mice fed a selenium-deficient diet (143). Moreover, there are modest qualitative differences in neurological dysfunction observed between the two genotypes (162).

The results of these studies point to roles for both domains of Sepp1 in the brain, although the C-terminal domain is clearly the major one maintaining the brain selenium content (143). The brain appears to depend on at least two Sepp1 functions: one is for Sepp1 in plasma to transport selenium to the brain from the liver and other tissues and the other is for Sepp1 synthesized within the brain to preserve brain selenium content (143). Contrary to previous studies our data show that plasma Sepp1 transported selenium to the brain is not required for normal hippocampal-dependent synaptic function.

Taken together, these data demonstrate that the conditional rescue of Sepp1 expression restricted to only forebrain neurons in Sepp1$^{-/-}$ mice is sufficient to reverse severe LTP deficits. These studies also present the excellent utility of this cell specific conditional genetic rescue model for studying the local and independent functions of other genes. These results support our hypothesis that Sepp1 has a significant role in local brain function, modulating synaptic plasticity, independent of hepatocyte-derived Se biosynthesis and transport. This study has successfully demonstrated that localized expression of Sepp1 in hippocampal neurons is necessary and sufficient for restoration of normal LTP, independent of Sepp1 transported from hepatocytes.

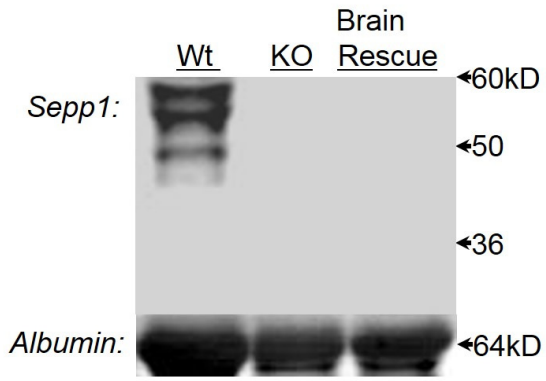


Figure 9. Sepp1 protein is absent in Sepp1 Brain Rescue sera. Sepp1 WT, KO, and Brain Rescue mouse sera shows that Sepp1 protein is not expressed in sera of Sepp1 Brain Rescue mice**.** Albumin, approximately 65kD, was used as loading control and visualized by Simply Blue SafeStain.

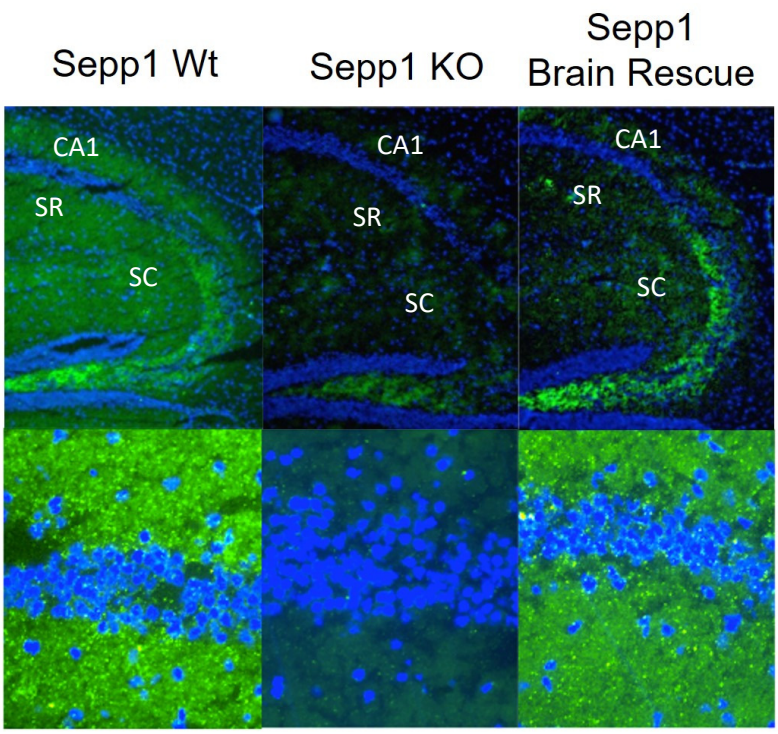


Figure 10. Sepp1 Brain Rescue forebrain neurons have restored Sepp1 protein expression. Immunofluorescence using Sepp1 primary antibody and Alex-fluor 488 secondary antibody (green) show that Sepp1 Brain Rescue have restored Sepp1 protein expression in hippocampal CA1 area similar to that observed in Sepp1 WT sections, while Sepp1 KO is confirmed to lack Sepp1 signal. DAPI was used to label nuclei in blue. SC = Schaffer collaterals, SR = stratum radiatum

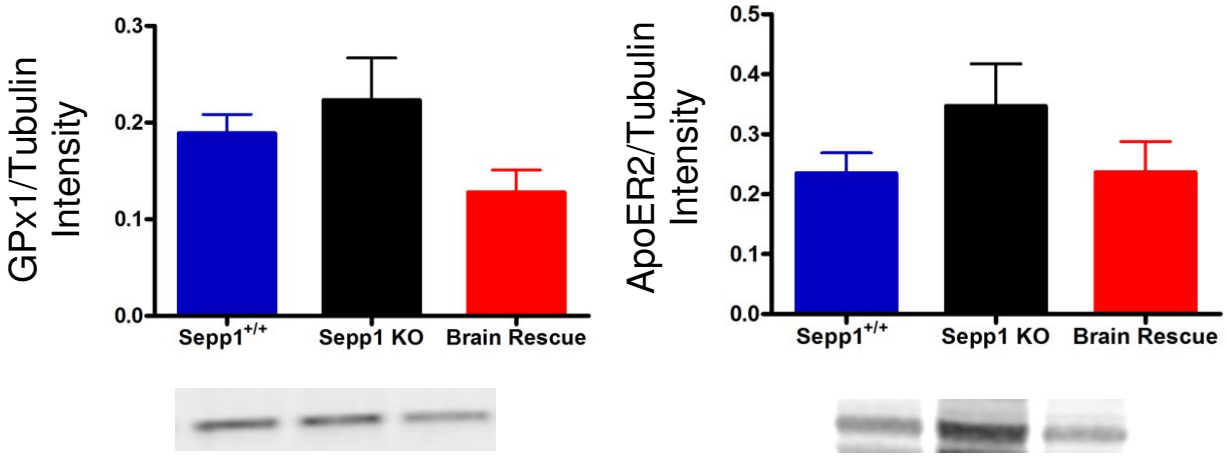


Figure 11. Neither Sepp1 deletion nor conditional forebrain-specific Sepp1 genetic rescue significantly alter GPx1 or ApoER2 expression in the hippocampus. A) Differences in GPx1 protein expression levels were not statistically significant by one-way ANOVA with Bonferroni Post-hoc test analysis ($p>0.05$). B) Sepp1 Brain Rescue ApoER2 protein expression is similar to that of Sepp1 WT mice, downregulating ApoER2 upon binding. Sepp1 KO mice show greater ApoER2 protein expression due to absence of Sepp1 receptor binding, but not statistically significant by one-way ANOVA with Bonferroni Post-hoc test analysis ($p>0.05$) (n=3-5 mice for each group for all protein analysis).

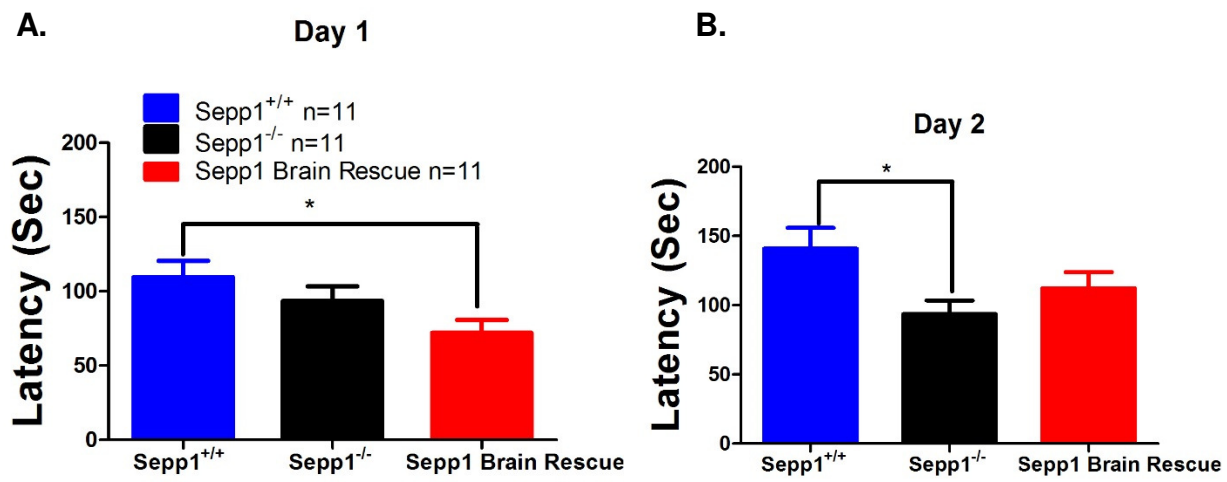


Figure 12. Sepp1 Brain Rescue mice exhibit marginally impaired balance and motor coordination. A) Rotarod performance on Day 1 showed Sepp1 Brain Rescue latency to fall was significantly greater than that of Sepp1 WT (one-way ANOVA with Tukey's multiple comparison post hoc test analysis, $p < 0.05$). The ability of Sepp1 KO mice to remain on the rod was less than that of Sepp1 WT on both days ($p > 0.05$). B) Sepp1 Brain Rescue mice demonstrated improved motor coordination and balance on Day 2 as compared to Day 1. Sepp1 Brain Rescue mice latency to fall did improve relative to the Sepp1 KO mice however still not as well as Sepp1 WT mice. Sepp1 KO performance on Day 2 did not improve as the other two groups did and was significantly poorer than that of Sepp1 WT (one-way ANOVA with Tukey's multiple comparison post hoc test analysis, $p < 0.05$). All groups showed improved latency on day 2 (2-way ANOVA, $p < 0.0001$).

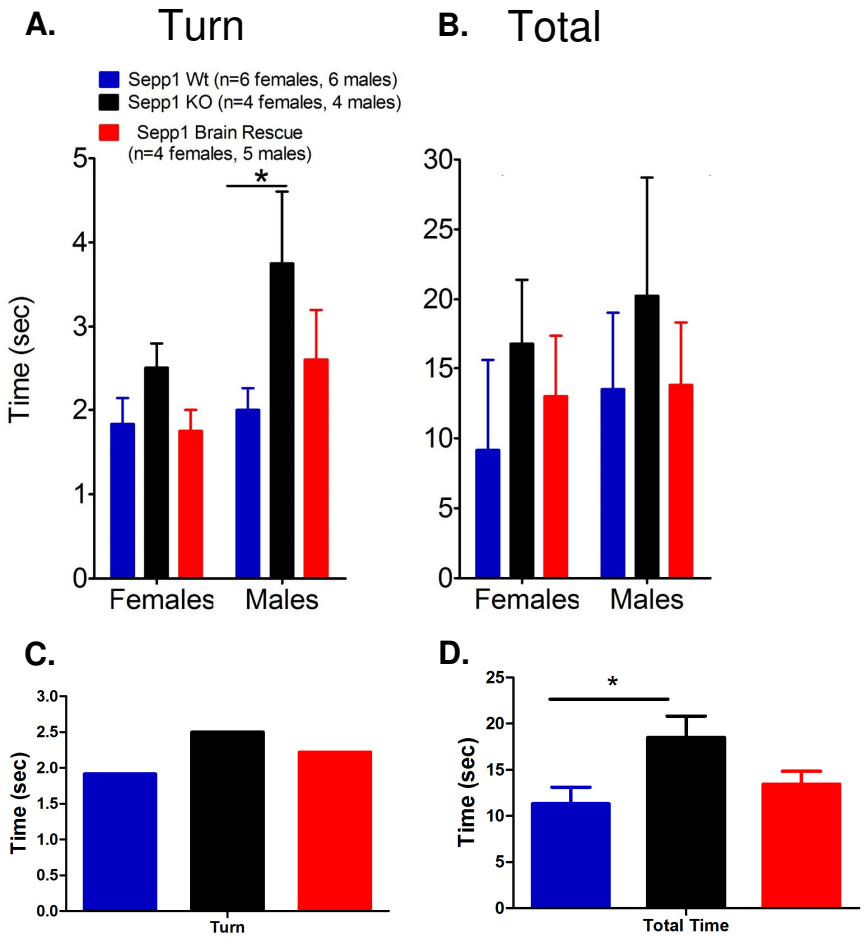


Figure 13. Sepp1 Brain Rescue exhibit moderate improvement of motor coordination on the Pole Test. (A) Time taken for Sepp1 Brain Rescue mice to coordinate and turn on the pole before descending was similar to normal Sepp1 WT mice in both females. Sepp Brain Rescue males performed slightly (not significant) worse than Sepp1 Brain Rescue females. Sepp1 male KO mice performed significantly worse than Sepp1 WT controls, two-way ANOVA with Bonferroni's Post hoc analysis performed for both (*$p<0.05$). Values are expressed as mean ± SEM. (B) Total time taken for Sepp1 Brain Rescue mice to descend the pole was similar to Sepp1 wild type (WT) in both females and males although Sepp1 Brain Rescue show slightly slower total descent time than Sepp1 WT controls but faster than Sepp1 KO mice. Sepp1 KO males performed worse than Sepp1 WT male controls, but only slightly worse than Sepp1 KO female counterparts ($p>0.05$), Sepp1 Brain Rescue ($p>0.05$) as reflected in two-way ANOVA results. (C) Grouped data of turn time, ($p>0.05$) by one-way ANOVA (D) Grouped data of total descent time, (*$p<0.05$) one-way ANOVA with Bonferroni's Post hoc analysis.

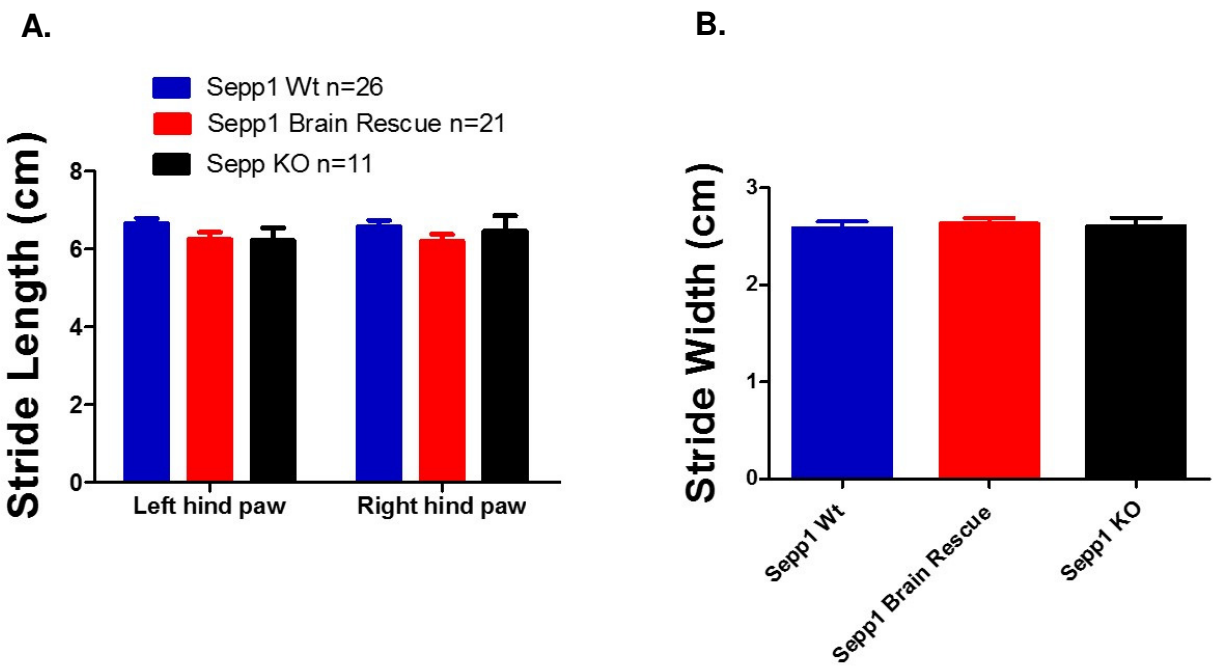


Figure 14. Sepp1 Brain Rescue mice have normal gait. (A) Sepp1 Brain Rescue (Sepp1$^{-/-}$CaMKII) mice exhibited normal and uniform stride length compared to Sepp1 WT mice gait in the stride test. 2-way ANOVA of stride length with Bonferroni post hoc analysis showed no significant differences in stride length or width between genotypes ($P>0.05$).

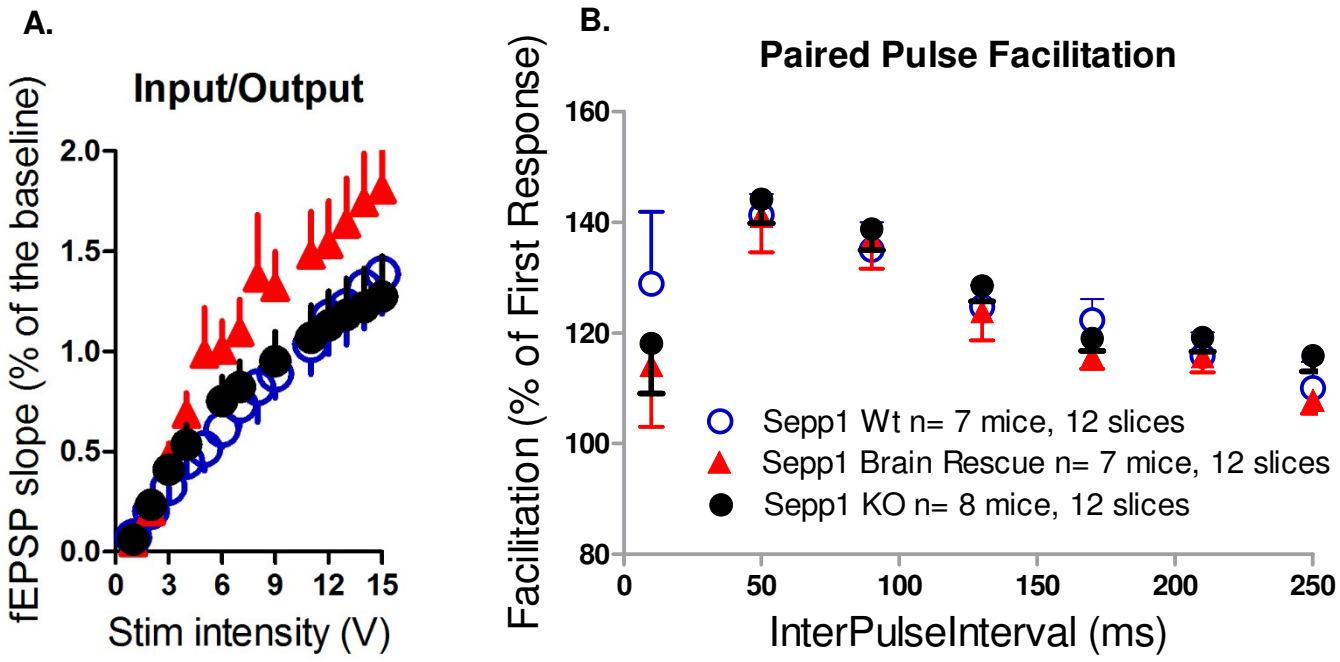


Figure 15. Sepp1 Brain Rescue mice exhibit normal synaptic strength and paired pulse facilitation. A) Input-output relationship of the slope of CA1 field excitatory postsynaptic potentials (fEPSP) in response to increasing stimulation of Schaffer collateral fibers appear increased with higher stimulus strength in Sepp1 Brain Rescue relative to Sepp1 WT and KO, but these changes in synaptic strength observed are not statistically significant via 2-way ANOVA. B) Sepp1 Brain Rescue mice have normal PPF, acquired by increasing inter-pulse intervals, compared to wild type counterparts ($p>0.05$ two-way ANOVA).

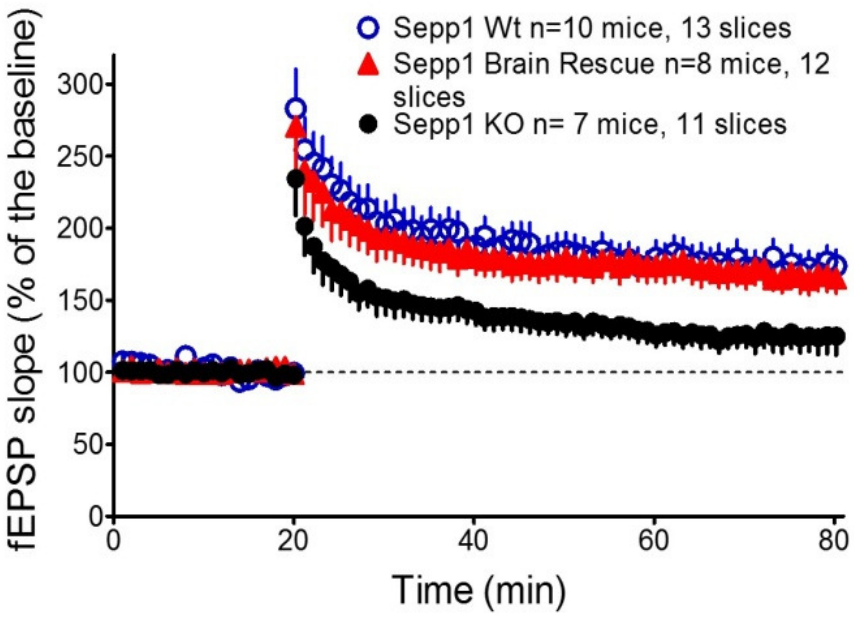


Figure. 16. **Sepp1 Brain Rescue mice exhibit restored long-term potentiation (LTP).** When Sepp1 expression is rescued only within forebrain neurons, LTP is restored, whereas Sepp1 KO mice are deficient in LTP, compared to wild-type mice ($p<0.05$, two-way ANOVA with Bonferroni posthoc tests). LTP was induced by high-frequency stimulation (HFS) (two 100 Hz trains, 20 sec apart).

Chapter IV

THE ROLE OF SEPP1 IN MODULATING THE DISABLED-1 PATHWAY

Abstract

Genetic deletion of Sepp1 in rodents leads to impaired neurological function, including abrogated synaptic plasticity (216), yet there are few published studies examining the mechanism and pathways in which Sepp1 is involved. Furthermore, while Sepp1 binds ApoER2, no studies have reported the role of Sepp1 in the signaling pathways associated with ApoER2. Mice with ApoER2 deletion on selenium deficient diets showed similar neurological deficits to those of the Sepp1 KO mice (103). The results imply that interruption of selenium supply evidenced by decreased selenium uptake to the brain leading to neurological deficits was due to impairment of the Sepp1-ApoER2 pathway (157,286). We hypothesize that Sepp1 affects the Disabled-1 pathway (Dab1) as Dab1 is a downstream effector that is activated by phosphorylation upon ligand binding to ApoER2. Both Sepp1 and Dab1 are required for normal synaptic plasticity (271).

Introduction

Disabled-1 (Dab1) is critical for synaptic function and learning. Dab1 is a downstream cytoplasmic adaptor protein of the Reelin-ApoER2 signaling pathway. Sepp1 shares the ApoER2 receptor with Reelin, a large extracellular matrix glycoprotein expressed by GABA-ergic interneurons (273). However, ApoER2 has a greater affinity for Sepp1 than Reelin (287). Reelin binds to the very-low-density lipoprotein receptor (VLDLR) and ApoER2 on the surface of post-mitotic neurons that migrate to their proper position in the developing brain (2, 5), leading to phosphorylation of Dab1 via binding at an NPxY consensus motif in the PTB domain on the cytoplasmic tails of the receptors and is a key regulator of Reelin signaling (288–291). Either Apoer2 or Vldlr alone is sufficient to induce Dab1 tyrosine phosphorylation in vitro (291,292). However, both both receptors are necessary for normal neuronal migration and in vivo Reelin-dependent enhancement of synaptic transmission in the hippocampus (273,292,293).

ApoE receptors have been indirectly implicated in memory and neurodegenerative disorders because their ligand, ApoE, is genetically associated with Alzheimer disease (273). Thus, the impairment of ApoE receptor-dependent neuromodulation may contribute to cognitive impairment and synaptic loss in Alzheimer disease (273).

Both ApoER2 and VLDLR belong to the LDL receptor gene family, which are a class of structurally closely related, yet unusually versatile, multifunctional endocytic cell surface receptors that regulate a wide range of cellular signaling pathways (292,294). These two receptors, together with their ligand Reelin, are key elements in neuronal migration and positioning during brain development (295,296). In this stage, reelin expressed by Cajal-Retzius cells (296–298) facilitates the correct development of different regions of the CNS, including the hippocampus, cerebellum, and cerebral cortex (296) . In the adult stage, the functions of ApoER2 and its ligand reelin include the regulation of synaptic plasticity (288,292,296), dendritic branching (296,299), actin remodeling, and neuronal survival (296,300). When oligomeric Reelin binds to ApoER2 and VLDLR, this initiates clustering of ApoER2 and VLDLR at the plasma membrane, inducing the phosphorylation of Dab1, which is a cytoplasmic adaptor protein (301). Oligomerization of Dab1 leads to phosphorylation of Dab1 (302). Opto-Dab1 may also be useful to study Reelin-Dab1 signaling in synaptic plasticity (302).

When phosphorylated on tyrosine, Dab-1 (mouse) forms complexes with proteins through a phosphotyrosine binding (PTB) domain (296,303). Dab1 is tyrosine phosphorylated by SFKs, and the kinases themselves can be further activated by phosphorylated Dab1 (304). Fyn-deficient mice showed increased Dab1 protein expression which suggests a response to impaired Reelin signaling similarly observed in mice lacking Reelin or its receptors (304). In Fyn-deficient neurons, upregulation of Dab1 expression is consistent with a response mechanism by which the cell attempts to compensate for reduced kinase availability (304).

Activation of Dab1 subsequently leads to activation of Src family tyrosine kinases (SFKs) and other kinases that phosphorylate the adaptor protein at its tyrosine residues (288). Defects in these signaling cascades as in apoER-2-deficient mice cause abnormal layering of neurons in the cortex, hippocampus, and cerebellum (9). Recent studies have shown that Dab1 is a critical regulator of synaptic function and

hippocampal-dependent associative and spatial learning in an adult forebrain-specific and excitatory neuron-specific conditional knock-out mouse line (271).

Reelin also modulates synaptic plasticity and learning in the adult mouse brain (205,273). Reelin has been shown to enhance long-term potentiation (LTP), but this function is abolished if either VLDLR or ApoER2 is absent (205). In this study, Reelin was found to be a potent enhancer of glutamate-stimulated Ca^{2+} influx through NMDA receptors (205). This Reelin-mediated modulation of NMDA receptor functions requires SFK and Dab1(205,301). Activation of PI3K A is critical in the Reelin signaling network (305,306), which leads to the phosphorylation of PKB/Akt (291,306) and activation of cofilin via LIMK1 (306). Reelin induces a complex network of events, the most prominent of which is the activation of PI3K. As a consequence, activated Akt regulates phosphorylation of tau, MAP1B mediated microtubule remodeling, as well as cell proliferation and survival (306).

$Sepp1^{-/-}$ mice have impaired synaptic plasticity as shown by deficits in LTP. Since Sepp1 knockout mice lack the SEPP1 gene, and thus cannot express Sepp1, Sepp1 binding to its receptor ApoER2 will not occur. As described when a ligand such as Reelin binds to ApoER2, it initiates a cascade of actions, subsequently inducing phosphorylation of Dab1. Therefore, we hypothesize that if Sepp1 acts similarly to Reelin upon binding to ApoER2, Sepp1 should induce phosphorylation of Dab1 upon binding. Thus we further hypothesize that phosphorylation of Dab1 will occur in the presence of Sepp1-induced LTP, while absence of Sepp1-induced LTP will result in either abolished or decreased Dab1 phosphorylation.

Peters et al. in the Weeber Lab at the University of South Florida, has shown that addition of 2 nM purified Sepp1 protein to Sepp1 KO hippocampal slices for only 20 minutes prior to HFS recovers LTP (307). However, in $Sepp1^{+/+}$ hippocampal slices, 2 nM purified Sepp1 protein blocked LTP, whereas 0.5 nM Sepp1 enhanced LTP (307). Interestingly, addition of Sepp1 purified protein to wild type slices decreases synaptic transmission and blocks Reelin binding (307). We hypothesize that Sepp1 modulates the Dab1 pathway working concertedly to regulate synaptic plasticity.

Materials and Methods

Sepp1 purified protein in concentrations of 0.25 nM and 2 nM were applied to hippocampal slices of Sepp1$^{+/+}$ and Sepp1 KO mice for 30 minutes to 1 hour. Negative control hippocampal slices were exposed to only ACSF. Positive control slices were exposed to pervanadate, a protein tyrosine phosphatase inhibitor that induces Dab1 phosphorylation. Briefly, whole brains harvested from Sepp1$^{+/+}$ and Sepp1 KO mice on standard lab diets were anesthetized with 2-2-2-tribromoethanol. Hippocampal slices will be prepared as previously described in Chapter 3. We measured phosphorylation of Dab1 in all hippocampal slices by protein analysis using an antibody to detect phosphorylated Dab1.

Results

Sepp1 can differentially modulate phosphorylation of Dab1 in a concentration-dependent manner (Fig. 17). Our data indicate a correlation between Sepp1 induced Dab1 phosphorylation and LTP. The protein analysis shows phosphorylation of Dab1 upon addition of 2 nM and 0.5 nM Sepp1 purified protein to hippocampal slices ex vivo for 40 Sepp1$^{+/+}$ slices exposed to 0.5 nM Sepp1 protein induced Dab1 phosphorylation and correlates to the enhanced LTP data by Peters et al (in preparation). However, application of 2nM Sepp1 protein, which has been shown to inhibit LTP, may result in much lower Dab1 phosphorylation. Sepp1$^{+/+}$ and Sepp1 KO hippocampal slices were exposed to 1 mM pervanadate which was the positive control as it is a general protein tyrosine phosphatase inhibitor (308). Greater phosphorylation of Dab1 was observed in Sepp1 WT slices than in Sepp1 KO slices with pervanadate treatment as expected due to absence of Sepp1. Pervanadate, a positive control, is a typically utilized broad protein tyrosine phosphatase (PTP) inhibitor to induce Dab1 phosphorylation (309,310). These data support our hypothesis and provides evidence that Sepp1 may modulate Dab1 phosphorylation, potentially similar to that of the Reelin-ApoER2 pathway.

Discussion

Apoer2 and Dab1 are present in the postsynaptic density where Apoer2 resides in a complex with NMDA receptors. Reelin, in conjunction with regulated alternative splicing

of ApoE receptors, regulates synaptic function, learning, and memory in the adult brain. (292). Reelin-mediated enhancement of LTP depends upon the presence of the 59 amino acids that are encoded by the alternatively spliced exon 19 (ex19). This exon is also necessary for the stimulation of tyrosine phosphorylation of NMDA receptor (NR2) subunits in hippocampal slices in response to Reelin. ApoE receptors have been indirectly implicated in memory and neurodegenerative disorders because their ligand, apoE, is genetically associated with Alzheimer disease (273). Thus, the impairment of apoE receptor-dependent neuromodulation may contribute to cognitive impairment and synaptic loss in Alzheimer disease (273).

In a study examining the role of Reelin in synaptic plasticity, Reelin was found to regulate synaptic plasticity in the adult brain and both of its receptors, VLDL receptor and apoER2, are necessary for Reelin-dependent enhancement of synaptic transmission in the hippocampus (273). Deletion of either VLDL receptor or ApoER2 resulted in the mice exhibiting contextual fear conditioning deficits (273). Defects in long term potentiation (LTP) were observed to be moderate in VLDL receptor-deficient mice and more pronounced in ApoER2 knockout mice (273). This study also showed that Reelin purified protein perfusion of mouse hippocampal slices significantly enhanced LTP in area CA1 and had no effect on baseline synaptic transmission (273). This Reelin-dependent enhancement in LTP was absent in VLDL receptor and apoER2 knockout mice. Hippocampal slices taken from conditional Reelin KO mice, 7 months of age, displayed elevated LTP. However, previous studies indicated that Reelin$^{-/-}$ mice had a reduction in LTP from outset, whereas direct injection of Reelin into the brain enhanced LTP (273). The results of the study suggested that elevated LTP in the conditional Reelin$^{-/-}$ mice represented a compensatory mechanism unique to the loss of Reelin in adulthood (273). Although Sepp1 is one of the most well investigated members of the selenoprotein family, its mechanism of action in maintaining synaptic plasticity remains unclear. From these previous studies, taken together with current knowledge of Sepp1 as a modulator of synaptic plasticity sharing the same receptor as Reelin, we surmise that the mechanisms in which Sepp1 modulates LTP may be similar to that of Reelin. We propose that Sepp1 through binding to ApoER2 induces phosphorylation of Dab1 and similar downstream signaling pathway as Reelin.

The Weeber Lab, our collaborator, show that treatment of Sepp1$^{-/-}$ mouse hippocampal slices with specific doses of Sepp1 purified protein for only 20 min prior to HFS could recover the LTP deficit normally seen in Sepp1$^{-/-}$ mice (307). Therefore, we applied Sepp1 purified protein in similar concentrations used in the Weeber Lab studies (0.5 nM, 2 nM) to hippocampal slices of Sepp1$^{+/+}$ and Sepp1 KO mice for 40 minutes. Our data show correlation between Sepp1 induced Dab1 phosphorylation and LTP. The differences in phosphorylation of Dab1 was observed in control treatment with ACSF in Sepp1$^{+/+}$ slices and Sepp1 KO slices were expected (Fig. 17D). The pervanadate treatment was used to induce Dab1 phosphorylation. Sepp1 slices in ACSF without Sepp1 purified treatment was our control to observe pervanadate treatment on each genotype without addition of the treatment, the Sepp1 purified protein (Fig. 17C). The elevated levels of phosphorylated Dab1 in Sepp1$^{+/+}$ was expected due to the nature of endogenous phosphorylation of Dab1 either by Sepp1 and/or by endogenous Reelin. Whereas, our control for the Sepp1$^{-/-}$ were expected to have much less phosphorylation of Dab1 since Sepp1$^{-/-}$ is not present. Exposure of Sepp1$^{-/-}$ slices to 2 nM Sepp1 purified protein induced Dab1 phosphorylation, which correlates with data from Peters et al., that LTP was recovered at this concentration (Fig. 17A). Potentially 2nM concentration may be a saturation point in which it is sufficient or this concentration may result in activating compensatory mechanisms. Conversely, 2nM in Sepp1$^{+/+}$ slices may have been over-saturating as the presence of Sepp1 increases endogenously phosphorylated Dab1 thus additional Sepp1 at this concentration may have ablated the response which would relate to a loss of LTP (Fig. 17A,D). Sepp1$^{+/+}$ slices exposed to 0.5 nM Sepp1 protein induced Dab1 phosphorylation (Fig 17B,D) and correlates to the enhanced LTP shown by Peters et al (307). Sepp1$^{+/+}$ and Sepp1 KO hippocampal slices were exposed to 1 mM pervanadate which was the positive control as it is a general protein tyrosine phosphatase inhibitor (Fig. 17C,D) (308). These new findings support our hypothesis that Sepp1 modulates phosphorylation of Dab1 correlating with LTP data generated from our collaborators (307).

The concentrations of Sepp1 purified protein applied ex vivo to hippocampi of Sepp1$^{+/+}$ and KO on standard chow were based on unpublished data from Peters et al. (162,307). The Weeber Lab demonstrated that application of Sepp1 purified protein can

either inhibit or enhance hippocampal LTP in Sepp1 mice (307). Dab1 and Sepp1 are both required for synaptic plasticity (216,311). We hypothesize that Sepp1 phosphorylated Dab1 response will correlate with LTP patterns (307)

Pervanadate was used for a positive control to induce tyrosine phosphorylation. Inhibition of the protein tyrosine phosphatases (PTPs) has been shown to mimic certain aspects of signal transduction that are normally triggered by tyrosine kinase activation (312). Inactivation of PTPs by pervanadate not only increases tyrosine phosphorylation of proteins that are regulated by tyrosine phosphorylation (312). Fischer demonstrating that inactivation of protein tyrosine phosphatases by pervanadate produced robust tyrosine phosphorylation of cellular proteins (308,312,313). Pervanadate also have insulin-mimetic properties and inhibits by irreversibly oxidizing the catalytic cysteine of PTP1B (283). Dab1 has N-terminal PTB domain, which mediates its binding to an NPXY motif in the insulin receptor tail when it is tyrosine-phosphorylated in response to insulin (278,282). Pervanadate have been widely studied for their insulin-mimetic effects and are commonly used as general inhibitors of PTPs (283).

We determined how much Sepp1 purified protein is required for phosphorylation of Dab1. We predicted that $Sepp1^{+/+}$ slices exposed to 0.5 nM Sepp1 protein would have greater Dab1 phosphorylation than wild type mouse hippocampi exposed to only ACSF. $Sepp1^{-/-}$ mice would have decreased relative phosphorylation of Dab1 as compared to $Sepp1^{+/+}$ mice whether exposed to 1mM pervanadate (positive control) or only ACSF (negative control) (Fig. 17C). Pervanadate induces phosphorylation of Dab1. 2nM Sepp1 protein, which has been shown to inhibit LTP (307), may result in no or very little Dab1 phosphorylation.

We hypothesize that Sepp1 is required for phosphorylation of Dab1 and correlates with synaptic plasticity functions. This data support our hypothesis and demonstrate a preliminary novel finding that Sepp1 has a role in phosphorylation of Dab1 and may share not only a common receptor, ApoER2, with Reelin but also a similar signaling pathway. These data, although still preliminary, will provide insight to the currently unknown mechanism of Sepp1 actions in neurological function. These findings are critical in elucidating the mechanism of Sepp1, providing the framework towards

understanding how Sepp1 signal transduction can be regulated to maintain normal brain function.

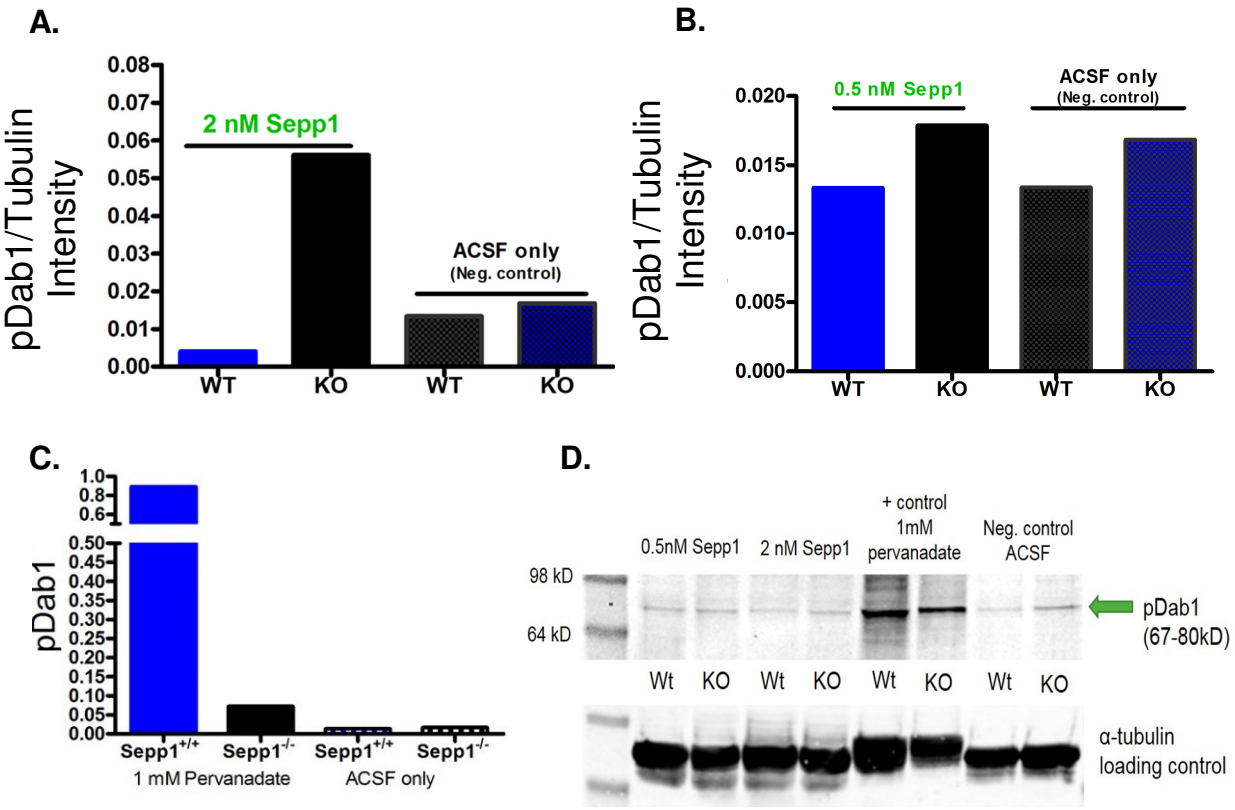


Figure 17. Sepp1 may induce phosphorylation of Disabled-1 (Dab1).

A) Phosphorylation of Dab1 induced by addition of 2 nM and 0.5 nM Sepp1 purified protein to hippocampal slices. B) Sepp1 KO slices exposed to 2 nM Sepp1 purified protein greatly induces pDab1 (reported to recover LTP- (307)). $Sepp1^{+/+}$ slices exposed to 0.5 nM Sepp1 protein induces Dab1 phosphorylation (enhanced LTP). C) $Sepp1^{+/+}$ slices exposed to pervanadate (positive control) and ACSF (negative control). D) Western blot showing protein expression of phosphorylated Dab1 in hippocampal slices treated with Sepp1 purified protein.

CHAPTER V

DELETION OF SELENOPROTEIN M LEADS TO GENDER-SPECIFIC SYNAPTIC PLASTICITY DEFICITS

Abstract

Selenoprotein M (SelM) is a small 15kD protein localized in the endoplasmic reticulum (ER) and contains the common redox motif of cysteine-X-X-selenocysteine type. SelM is highly expressed in brain and has been shown to have a potential role as an antioxidant, have neuroprotective properties, regulate cytosolic calcium, and most recently found to potentially be a biomarker for certain types of cancer (2,314). SelM has been linked to neurodegenerative disease models. SelM expression was suppressed by expression of a presenilin-2 mutation in a previous study of an Alzheimer's disease (AD) mouse model (3). In light of the expression profile of SelM, its neuroprotective properties, and association with AD pathology, we hypothesize that SelM has important roles in synaptic plasticity, learning and memory. We report that SelM KO male mice are deficient in hippocampal-dependent LTP, a cellular model for learning and memory, whereas SelM KO female mice exhibit normal LTP responses. Our novel findings demonstrate that genetic deletion of SelM has a gender specific effect on synaptic plasticity, an important aspect in maintaining normal hippocampal function, facilitating learning and synaptic plasticity.

Introduction

SelM is highly expressed in several regions of the mouse brain, including a relatively strong presence in the CA2 and CA3, and a relatively lower amount in the CA1 region of the hippocampus (2,100). SelM is also present in the paraventricular and arcuate nuclei of the hypothalamus, ventral tegmental area (VTA), red nucleus (RN), and Purkinje layer (PL) of the cerebellum, brain stem, the hippocampus, and cerebral cortex (100). The study also reported no morphological changes in the hippocampus or other regions of the brain through thionin staining of $SelM^{-/-}$ mice (100). As previously mentioned, SelM is been shown to be involved in ER calcium regulation in neurons. Although

several new studies have emerged in the last few years, the function of SelM in the brain still remains elusive. This study investigates whether SelM is required for hippocampal-dependent synaptic plasticity. We hypothesized that deletion of selenoprotein M (SelM) would alter hippocampal synaptic plasticity. To investigate this hypothesis, we employed electrophysiology techniques to assess long-term potentiation (LTP), a model for cellular learning and memory, in SelM KO and wild type control mouse hippocampal slices. This study will provide crucial insights on the role of SelM in maintaining normal synaptic function that will provide the framework for further studies exploring the role of SelM in neurodegenerative pathology.

Materials and Methods

Animals

Animals were provided food and water as needed according to the guidelines of the University of Hawai‘i Institutional Animal Care and Use Committee (IACUC). All animals in this study were maintained on standard laboratory diets containing ~0.25 ppm Se. This is considered Se-adequate but may be deemed to be slightly supplemented based on the rodent recommended daily allowance (RDA) of 0.15 mg/kg Se (113,315,316). Animals were kept on a 12-h light cycle and group housed during breeding and rearing. Prior to and during behavioral testing, animals were individually housed in polycarbonate cages. Each cage was provisioned with food, water, and a layer of bed-o-cob (corn cob) bedding (Newco Distributors). All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Generation of SelM$^{-/-}$ mice

Heterozygous SelM$^{+/-}$ mice were generated by the Knock-Out Mouse Repository (KOMP, University of California, Davis). Heterozygous SelM$^{+/-}$ mice were provided with drinking water supplemented with 10 µM sodium selenite since viability was low in the first litters of heterozygotes in the absence of Se supplementation. Breeders and dams were continued on Se-supplemented drinking water. SelM$^{-/-}$ pups were subsequently weaned from Se supplemented water to regular water. Experiments were performed on

age and sex matched adult C57BL/6 wild-type or SelM$^{-/-}$ mice 12 to 24 weeks of age on regular drinking water not supplemented with Se.

Immunohistochemistry

Animals were deeply anesthetized with 2-2-2-tribromoethanol, administered by intraperitoneal injection at a dose of 250 mg/kg (approximately 0.5 ml to a 25gm mouse) and perfused with phosphate buffered saline (PBS) containing 4% paraformaldehyde (PFA). Brains were excised and post-fixed in PBS with 4% PFA for 24 hours and immersed in graded solutions of sucrose (10%, 20%, 30%) until brains were saturated. Brains were then embedded in OCT and sectioned parasagittally into 40 µm sections with a Leica CM 1950 cryostat microtome and collected sequentially. Brain sections were stored in a cryoprotective solution (50% PBS, 25% glycerol, and 25% polyethylene glycol) at 40℃. For diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry, sections were then incubated in citric acid (0.01M, pH 6) (Trilogy) and heated to 95℃ for 15 min for antigen unmasking. After allowing 15 minutes for samples to cool at RT, sections were then washed in PBS and treated with 3% H_2O_2 in MeOH for 10 min to inactivate endogenous peroxidases. Tissues were then washed with PBS and incubated for one hour at room temperature with blocking solution 5% normal goat serum (NGS) in PBS-T (PBS containing 0.3% Triton X-100). Samples were then incubated for 15 minutes in Avidin Blocking Solution then wash briefly once with PBS-T and twice with PBS. Samples were further incubated with Biotin blocking solution for 15 min then washed once with PBS-T and twice with PBS. Subsequently sections were incubated overnight at 40℃ with rabbit anti-Selenoprotein M polyclonal antibody (Sigma Aldrich Co.) in 2.5% NGS in PBS-T. The following day, all sections were first washed with PBS-T. Brain sections were then incubated for one hour at room temperature with biotinylated anti-rabbit IgG secondary antibody per manufacturer directions. Sections were washed again with PBS-T, incubated for 30 minutes in ABC Solution (Vector Laboratories) (1:500 streptavidin-horse radish peroxidase. Section were washed with once with PBS-T for 5 min, then twice with PBS for 5 min each wash and developed with diaminobenzidine tetrahydrochloride using a VectorStain ABC kit (Vector Laboratories) per manufacturer's instructions. Immunoreactivity was visualized by

peroxidase detection with diaminobenzidine tetrahydrochloride acting as a chromogen. After several rinses in PBS, sections were mounted on slides, dehydrated with graded solutions of ethanol followed by xylene, and coverslipped.

Microscopy.

Mounted sections were viewed and images collected with a Zeiss Axioskop 2 Plus with a digital camera.

Hippocampus Slice Preparation and Field Potential Recordings

Acute hippocampal slice preparation and electrophysiology were performed in mice 12 to 24 weeks of age using standard methods previously described (5). Mice were deeply anesthetized with filtered 2-2-2-tribromoethanol (TBE) administered by intraperitoneal injection at a dose of 250 mg/kg (approximately 0.5 ml per 25gm mouse body weight). Brains were rapidly removed and quickly placed in ice cold artificial cerebrospinal fluid (ACSF: containing in mM: 130.0 NaCl, 3.5 KCl, 24.0 NaHCO3, 1.25 NaH_2PO_4, 1.5 $MgSO_4$, 2.0 $CaCl_2$) oxygenated with carbogen (95% O_2 5% CO_2). Transverse brain slices (350 µm) were cut using a microslicer (Leica VT1000S) while submerged in ACSF oxygenated with carbogen. Hippocampi were isolated from the slices while in pre-oxygenated ACSF, transferred to a holding chamber with a nylon mesh support and allowed to equilibrate at 32^{o}C in oxygenated ACSF. All slices were permitted a minimum 1 hr recovery time before transfer to a recording chamber, where the slices were continuously superfused with oxygenated ACSF at a rate of 1-3 ml/min. Extracellular field recordings were obtained from the stratum radiatum of the CA1 area. Field EPSPs (fEPSPs) were evoked by stimulation to the Schaffer collaterals and recorded with a concentric bipolar stimulating electrode. Input-output curves were obtained by varying stimuli from 1 to 15 V in 1 V increments. Paired-pulse facilitation was achieved with pairs of stimuli at 3V with interstimulus intervals of 10, 50, 90, 130, 170, 210 and 250 msec, with 30 sec between each pair of stimuli. Long-term potentiation (LTP) was induced with high-frequency stimulation (HFS) following at least 20 min of stable baseline recording. HFS consisted of two trains of 100 Hz frequency stimulation for 1 sec duration with a 20 sec interval each train. Stimulation (3V) was

used to produce fEPSPs that were approximately 10-30% of maximum responses. Potentiation was measured as the increase of the mean pEPSP following HFS normalized to the mean pEPSP for the duration of the baseline recording.

Data acquisition

Extracellular field recordings were acquired using an Axoclamp 900A microelectrode amplifier (Molecular Devices). The signal was filtered at 1 kHz, digitized at 10 kHz, and stored on a personal computer equipped with Axograph Scientific Software (Axograph X). The amplitude of the EPSP was measured as a peak value. The fiber volley amplitude was measured as a difference between the initial positive and the following negative peak.

Statistical analysis

See Chapter II and III.

Results

The expression pattern of SelM in the mouse brain. First, to verify expression of SelM in the hippocampus, immunohistochemistry was performed on parasagittal brain sections of wildtype and SelM$^{-/-}$ mice using an anti-SelM antibody. A previous study reported, based on the Allen Brain Atlas, that SelM mRNA levels are highest in the hippocampus, olfactory bulb, and cerebellum (100,317). More recently, another study conversely reported that SelM protein levels were moderate in the hypothalamus, but relatively low in the hippocampus compared to other regions (100). Specific to the hippocampus, the study noted that SelM expression was highest in the CA2 and CA3, and least in the CA1, the region central to our synaptic plasticity study (100). The SelM antibody used in our group is known to require high concentrations for detection. We used an antigen unmasking protocol to reduce the non-specific binding due to protein-protein crosslinking, but some relative background remains, potentially from the fixation process which causes protein-protein crosslinking. Nonetheless, we show that SelM is

indeed relatively expressed in the CA1 region of the hippocampus, the region of interest in these studies compared to the SelM KO (Fig.18).

Deletion of SelM in male mice alters synaptic transmission and long-term potentiation. We examined LTP in hippocampal slices isolated from brains harvested from $SelM^{+/+}$ and $SelM^{-/-}$ mice to determine if deletion of SelM leads to alterations in synaptic plasticity. The initial input-output of synaptic connections in the Schaffer collateral-CA1 presynaptic fibers were measured using increasing stimulus intensities and recording the evoked CA1 field excitatory postsynaptic potentials (fEPSPs) in the stratum radiatum at the Schaffer collateral fibers. $SelM^{-/-}$ male mice exhibited an overall increase in synaptic efficacy significantly shown by an augmented input-output relationship of the fEPSP ($P<0.05$, linear regression, two-way ANOVA) (Figure 19A). $SelM^{-/-}$ females exhibited normal basal synaptic efficacy compared to wild-type counterparts. We then analyzed the relationship between the evoked fEPSP slope and the corresponding fiber volley amplitude, which is a measure of presynaptic depolarization. We determined that slices from $SelM^{-/-}$ male mice exhibited a greater increase in fEPSP slope than those from SelM male, female wild-type, and also compared to $SelM^{-/-}$ female mice suggesting $SelM^{-/-}$ male mice have increased afferent synaptic function in the CA1 of the hippocampus (Fig. 19B).

Paired-pulse facilitation (PPF) is a well-studied form of short-term synaptic plasticity in which two presynaptic stimuli are administered in close succession. PPF is primarily a measure of presynaptic function, but could induce postsynaptic changes. The resulting fEPSP responses to pairs of stimuli are measured as the ratio of the slope of the second evoked fEPSP over the first fEPSP. The $SelM^{-/-}$ male slices exhibited a slight decrease in PPF compared to wild-type male slices but was not significant. We observed no significant difference in PPF in $SelM^{-/-}$ male or female mice, arguing against any presynaptic changes (Fig.19C).

We investigated synaptic plasticity in the form of long-term potentiation (LTP), a cellular model for learning and memory. LTP was induced by high frequency stimulation (HFS) consisting of two trains of HFS (100 Hz, 1 sec) spaced 20 seconds apart. Interestingly, slices from $SelM^{-/-}$ male animals have severe deficiency in LTP compared

to wild-type counterparts (P=0.0006) (Fig. 20). Although the increases in fEPSPs were similar between groups immediately following HFS, and induction was not significantly different, potentiation in SelM$^{-/-}$ male animals were significantly lower than slices from wild-type animals starting from 6 minutes after HFS until end of recording (P<0.001 to 0.05). Interestingly however, SelM$^{-/-}$ females animals did not have any change in LTP compared to their wildtype counterparts as seen in the SelM$^{-/-}$ males animals. We determined that the deficit in LTP of SelM$^{-/-}$ male mice are significantly diminished compared to SelM$^{-/-}$ female mice (p<0.001 to 0.05, two-way ANOVA with Bonferroni's posthoc tests) (Fig. 20). Taken together, our data shows that genetic deletion of SelM has a gender specific effect on LTP, negatively impacting synaptic plasticity in SelM$^{-/-}$ male mice.

Discussion

The function and mechanisms of SelM in the brain regarding learning and memory formation has not been as well studied as Sepp1. The current breadth of knowledge asserts that SelM plays an important role in antioxidant defense, is in involved in ER calcium signaling, and may have a protective role in AD pathology. In this study, we provide evidence that SelM does have a role in the molecular basis of memory formation. We also show that SelM deletion effects the deficits in synaptic plasticity in gender specific manner. The altered synaptic plasticity observed in SelM KO male mice provides novel insights into the importance of SelM function in the brain. These studies offer mechanistic insight to the neuroprotective role of SelM in the brain.

Initially we hypothesized any alterations in synaptic plasticity would affect both SelM$^{-/-}$ male and females as there were no significant gender differences reported in early characterizations of SelM$^{+/+}$ or SelM$^{-/-}$ mice. Interestingly, however, data from our electrophysiology results reveal a gender difference in which only SelM$^{-/-}$ male but not SelM$^{-/-}$ female are deficient in LTP due to genetic deletion of SelM. Additionally, male SelM$^{-/-}$ mice had increased synaptic efficacy, which is unlikely to be caused by an increase in neurotransmitter release, as indicated by the lack of change in PPF in male SelM$^{-/-}$ mice. Many studies show that PPF is used as an indicator of a possible change in presynaptic release probability, due to astrocyte calcium signaling (2, 3). Based on

previous reports that SelM is involved in calcium regulation (23), there may be an association of these mechanisms.

In a study further contributing to characterizing the neurobiological role of SelM, Pitts et al., determined that SelM$^{-/-}$ mice did not have any significant neurobehavioral impairments (318). This study employed a series of neurobehavioral assays to further characterize the effect genetic deletion of SelM compared to SelM$^{+/+}$ mice, including open field test and elevated plus maze (mice receive one trial up to 5 minutes) which assess anxiety, general locomotion, motor coordination; Rotorod test (motor learning and coordination); Barnes Maze which is similar to the well-known Morris Water Maze (MWM), except it assesses spatial learning and memory on dry land and not in water; and fear conditioning (100). Memory deficits could be progressive over time as the animals age, a phenomenon that occurs in humans. Therefore it also possible that the animals that developed the altered metabolic (5-6 months) were just beginning to exhibit degenerative pathology whereas the mice subjected to the Barnes maze were slightly younger animals (4-5 months). As both neurodegeneration and metabolic disorders develop progressively, it would also be prudent to examine whether age correlates with development of disease pathology in SelM mice. We also posit that the synaptic plasticity deficit in SelM$^{-/-}$ male mice may not be associated with the specific types of neurobehavioral function assessed by the paradigms employed in previous studies.

Although LTP is a cellular model for learning and memory and is often positively correlated with results from neurobehavioral paradigms, the phenomenon of LTP is not universal in all learning experiences (319). The NMDAR subunit NR2A was shown to be required for rapidly acquired spatial working memory (SWM) but not incremental spatial reference memory (SRM) (320). Mice with deletion of NMDAR subunit NR2A were able to perform similarly well in the MWM, which test for SRM. The radial maze (also a SRM test) results confirmed the same finding as did the MWM test, that the NR2A$^{-/-}$ mice also performed as well as wildtypes. The elevated T-maze (a type of elevated plus maze paradigm) was used as a spatial non-matching-to-place test. This hippocampus-dependent, discrete-trial, rewarded alternation task assay is typically administered to assess SWM. This version, quite different than the elevated plus maze protocol to test for general anxiety which exposes the mice once for 5 minutes (100,321), is described

in detail by Bannerman et al (320). Briefly, it requires mice requires the mice to do two runs, a sample run where the mouse is placed in the start arm of the T-maze and allowed to enter a goal arm (the other arm is blocked) followed by removal of the mouse for a specified delay period. After the delay, the mouse is returned for the choice run (no arms are blocked) (320). Wild-type mice showed exceptional alternation, but NR2A$^{-/-}$ mice displayed a decreased level of SWM performance. (320). The authors who conducted a battery of other behavioral assays, did state the MWM results were different from previous reports, as it is believed that spatial learning task such as finding the hidden platform in the MWM is essential in NMDAR-dependent LTP. Their results establish a distinction that NR2A is required for spatial working memory not spatial reference memory indicating that specific types of memory require specific receptor subgroups.

NMDARs, heteromultimeric ion channels and a glutamate receptor (GluR), serve critical functions in the brain and act as primary mediators of Ca^{2+} influx during synaptic activity and thus during LTP (see Synaptic Plasticity, p.98) (322). The NMDA receptor channel is formed by the GluR epsilon (NR2) and GluR zeta (NR1) subunit families (323). The GluR epsilon are comprised of epsilon 1 (NR2A) and epsilon 2 (NR2B) (323). In a different study, NR2A$^{-/-}$ (NR2A also known as GluRε1) mouse model showed that mice exhibited a reduction in hippocampal LTP and were normal in fear conditioning and contextual fear conditioning testing (324). However, upon stronger tetanic stimulation, NR2A$^{-/-}$ LTP deficit was restored and the saturation level of LTP was unaltered (324). The results of the Kiyama et al. study suggest an increase of threshold for LTP induction in the NR2A mutant mice was necessary to restore the deficits (324). These mice had normal responses to standard fear and contextual conditioning protocols, however when time interval between shocks and placement into chamber shortened, abnormal fear conditioning responses were observed, suggesting that NR2A is essential for threshold determinant for LTP and contextual fear conditioning. Another study reported that spatial memory consolidation as observed in the Morris Water Maze (MWM) task, specifically requires hippocampal dependent LTD (325). The study showed that mice that were given a in order to block LTP, and then subjected to the Morris Water Maze (MWM) task, had no significant negative effect on any aspect of

these mice performance in the MWM performing similar to mice not administered the LTP-blocker GluN2A (also known as NR2A) antagonist (325). Whereas mice given the LTD-blocking GluN2B (also known as NR2B) antagonist exhibited impaired spatial memory consolidation in the MWM task (325). This corroborates previous study that NR2A is specific for special working memory which may be best assessed by other assays and not the MWM. We show that SelM deletion does alter synaptic plasticity as LTP is deficient. Although SelM$^{-/-}$ mice does not exhibit spatial reference memory deficits determined by the MWM described in the studies cited here, SelM$^{-/-}$ mice exhibit similar absence of LTP but normal behavior in the MWM as do the NR2A$^{-/-}$ mice and also the mice given the GluN2A antagonist to block LTP.

Furthermore, studies with AD mice models have shown that an increased ryanodine receptor-evoked calcium signals within dendritic spine heads, dendritic processes, and the soma of pyramidal neuron. Synaptically-evoked postsynaptic calcium responses and calcium signals generated from NMDA receptor activation were larger in the AD strains. Ryanodine receptors are cellular mediator of calcium-induced calcium release (CICR) (326). LTP can be blocked by depletion of ER calcium stores, indicating that calcium release from intracellular stores is also critical for LTP induction (327,328). Currently our data show that SelM has deficiency in LTP, as well as previous reports that SelM has role in protecting neurons from oxidative damage through regulation of ER calcium stores, SelM knockdown increased baseline levels of cytosolic calcium, and is localized to the ER like other calcium regulators (2). Taken together, SelM, as a calcium regulator, which may contribute to synaptic plasticity function, may have a potential role in modulating NMDARs, specifically, NR2A, which is important as a threshold determinant for LTP therefore spatial working memory.

The mechanism by which SelM regulates synaptic plasticity and metabolic function most likely involve a variety of converging signaling pathways, which is complicated by differing regulations due to sexual dimorphism. SelM$^{-/-}$ mice were described to be obese and have gender specific metabolic dysfunctions. Selenium and many selenoproteins are involved in production of hormones, growth factors, and regulation of normal metabolic function (8). High expression of selenoproteins as well as selenoprotein deficiency may cause glucose homeostasis dysregulation (329).

Activation of thyroid hormone, which is involved in neuroendocrine homeostasis, is dependent on the DIO class of selenoproteins. Individuals with genetic defects in SECIS-binding protein 2 (SBP2) exhibit a syndrome of selenoprotein-related defects including abnormal thyroid hormone metabolism (330). Overexpressing cellular GPx induces development of insulin resistance and obesity (331). SelS, another ER protein similar to SelM in size and localization is glucose-regulated, and was originally discovered in a rodent model for diabetes (8,332). Sepp1 was significantly increased in circulating serum Sepp1 levels in patients with prediabetes and type 2 diabetes mellitus compared to those with normal glucose tolerance (333). Therefore it is not surprising that SelM$^{-/-}$ mice have defects in metabolic regulation.

SelM$^{-/-}$ animals were reported to be obese but had normal glucose tolerance, but low corticosterone levels in the morning (100). Only SelM$^{-/-}$ males had increased fasting insulin, while SelM$^{-/-}$ females had elevated corticosterone (in the evening) and p-Akt (insulin signaling in the liver) levels in females (100). When challenged with a Se deficient diet (0.01 ppm Se), SelM$^{-/-}$ mice was observed to decrease locomotion, females increased in body weight compared to their wildtype counterparts while SelM$^{-/-}$ males remained the same, with the greatest differences seen at 20 weeks (100) Levels of inguinal white adipose tissue had already increased twice as much as wildtype prior to Se diet challenge and remained elevated after diet challenge (100).

Many selenoproteins also have a gender specific effect such as Sepp1 in which Sepp1 and its known receptor ApoER2 are abundantly expressed in male testes but absent in female reproductive organs (47). Sepp1 is also closely related to glucose metabolism. In the NHANES III study which assessed serum selenium and diabetes in U.S. adults, only males with very high Se status were reported to have an increased risk of diabetes (334,335). In rodents, higher Sepp1 and deiodinase mRNA levels are observed in liver (336,337). Raman et al., reported that Sepp$^{-/-}$ male mice are more dependent on Sepp1 and Se than females for normal brain function (113). GPX3 is more highly expressed in healthy females than in males (338,339), and that alcohol consumption increased total Se in women but not in men (338,340). GPX3 activity increases in parallel to estrogens during the female menstrual cycle (338,341), and females express higher serum GPX3 concentrations than males, which is also seen in

rodents (338,339). The erythrocyte GPx activity was confirmed to be expressed higher in women than men confirming previous studies, which is likely due to high estrogen levels and hormonal contraceptive use (336,342). These studies support the theory that estrogens are responsible for the sex-related differences in glutathione peroxidase activity (343,344). A study examining hepatic expression of SepS in immune response found expression Se supplementation increased SepS levels and decreased the fulminant immune response in male mice raised on a selenium deficient diet whereas females were similar to wildtype (345). Se metabolism interaction with gender, body weight, and adiposity are complex. The effect of steroid hormones, as well as hormones and cytokines from adipose tissue are all dependent on sex. SelS similar to SelM is involved in responding to ER stress (8,345).

Metabolic disorders has been shown to contribute to synaptic plasticity dysfunction. The liver is the major insulin target organ responsible for control of glucose homeostasis in the fasting state (346,347). The hypothalamus-pituitary-adrenal axis responds to stress by signaling the increase of cortisol secretion. The hippocampus provides negative feedback to the HPA and plays a critical role in key aspects of spatial and declarative memory (348). Therefore, hippocampal dysregulation could contribute to both the memory impairment and neuroendocrine abnormalities found in neurological conditions (348). The findings of this current study that SelM$^{-/-}$ mice have synaptic plasticity deficits are potentially associated with the metabolic dysregulation such as the reported increase in insulin levels and having altered HPA activity. Furthermore, the gender effects in SelM$^{-/-}$ male mice appear to correlate with many of the other selenoproteins in which deletion or alteration leads to a more severe phenotype or health dysregulation in males, such as seen in Sepp1$^{-/-}$ male mice. We will further explain how the previously report metabolic dysregulation Sepp1$^{-/-}$ males have increased insulin levels compared to females support our data and contribute to SelM$^{-/-}$ LTP deficits in male mice

Earlier, we discussed that SelM may modulate NMDARs, specifically, NR2A as mice with NR2A deletion share similar hippocampal synaptic plasticity and behavioral phenotype as seen in SelM$^{-/-}$ mice. Here, we discuss the gender specific effect in which SelM deletion only alters synaptic function in male mice and how the metabolic

dysregulation such as elevated insulin levels in only male mice effect synaptic plasticity. SelM$^{-/-}$ females exhibited increased corticosterone activity and not males compared to wildtypes, but also had normal hippocampal dependent synaptic plasticity.

The metabolic system regulate insulin signaling as well as hormonal responses to physical and physiological stress, which can exert an effect on multiple functions in the body, such as synaptic plasticity and memory. However, stress does not lead to a universal suppression of LTP (349). Many factors, including the type of stress, the phase of the stress response, the area of investigation, type of LTP, and the life history of the organism determine in which direction LTP will be changed (349). Metabolic alterations can result in gender-specific synaptic plasticity deficits. In a study investigating the impacts of a high fat diet on obesity, metabolic and stress hormones, learning performance, and hippocampal synaptic plasticity, obese male, but not female, mice exhibited lower LTP and LTD at the Schaffer collateral-CA1 synapses of the hippocampal slices, as compared with their sex-specific controls. The study results suggest that male mice are more vulnerable than the females to the impacts of a high fat diet on weight gains, metabolic alterations and deficits of learning, and hippocampal synaptic plasticity (350).

Here we discuss how the unique metabolic and synaptic dysfunction profiles in SelM$^{-/-}$ male mice are shared in IRS-2 deficient mice, regarding LTP deficits, insulin signaling alterations in a gender-specific manner. Insulin, leptin, and adiponectin are important peripheral signals that advise the brain of short-and long-term nutrient availability (351–354). Insulin and its insulin receptors are localized to glutamatergic synapses in the hippocampus (355). Insulin-regulated trafficking of NMDARs may play a role in synaptic transmission and plasticity, including long-term potentiation (355). IRS-2 is highly expressed in the hypothalamus, as is SelM, and regulates brain growth, body weight control, glucose homeostasis, and female fertility (74). Its signaling cascade may be responsible for integrating central control of nutrient homeostasis and appetite regulation with peripheral insulin action and β cell function (351). IRS-2 is an adaptor protein that combines activation of insulin- and insulin-like growth factor-1-receptors to downstream signaling pathways (356). IRS-2 signaling appears to be more important than IRS-1, as IRS-1$^{-/-}$ mice does not alter total insulin-induced PI 3-kinase

activation (347,357–359). Patients with insulin resistance and metabolic syndrome have reduced IRS-2 expression (360–362) as also observed in animal models with insulin resistance in several insulin-sensitive tissues (347,363–365), suggesting that the low expression level of IRS-2 contributes to metabolic dysfunction. In a hepatocyte cell culture model, the effect was identical- insulin treatment downregulated IRS-2 mRNA and protein levels which was attributed to by the repression of IRS-2 gene transcription due to insulin, mediated by the PI 3-kinase/Akt pathway and by nuclear proteins binding to the IRE sequence on the IRS-2 gene (347). IRS-2 conditional brain knockout including the hypothalamus lead to increased appetite, lean and fat body mass, linear growth, and insulin resistance that progressed to diabetes (351).

Genetic deletion of IRS-2 has been shown to have a sexual dimorphism and is important in energy homeostasis (366,367). B6J-Irs2$^{-/-}$ male mice showed greater insulin resistance glucose tolerance impairment compared to wildtype, while B6J-Irs2$^{-/-}$ female mice had only slight impairments (368). This study observed that from 14 to 24 weeks of age, 20% of male B6J-Irs2$^{-/-}$ mice suddenly exhibited severe increase in mean blood glucose and died within a short time with polydipsia/polyuria and loss of body weight (368). IRS-2$^{+/-}$ mice had significantly increased fasting insulin levels compared with those for WT (361). IRS$^{-/-}$ male mice were overtly diabetic by 12 weeks of age, whereas female IRS-2$^{-/-}$ animals develop mild obesity and progress less rapidly to diabetes (347).

In IRS2$^{-/-}$ mice hippocampus slices, LTP induced by high-frequency conditioning tetanus failed to activate postsynaptic NMDA receptors. IRS$^{-/-}$ had normal responses in synaptic efficacy and pair-pulse facilitation therefore alterations are most likely post-synaptic (369). IRS2$^{-/-}$ mice exhibited defective activation of Fyn, AKT, and MAPK in the brain in response to tetanus stimulation (370). However, studies examining whole brain and forebrain-specific knockout mice show that behaviorally, they exhibit improved memory retention in the MWM test and whole brain knockout mice have enhanced spatial working memory and contextual- and cued-fear memory (356).

The deletion of SelM leading to the hippocampal synaptic plasticity deficit as we have shown and metabolic alterations previously reported may be due to both the role of SelM mediating oxidative stress, leading to a cascade of effects modulating NR2A.

SelM deletion may synergistically effect both expression of IRS-2 and NR2A which converges and in turn regulates synaptic plasticity. Metabolic dysfunction may contribute to the defect in synaptic plasticity as seen in other studies in which increase insulin signaling is one of many factors leading to alterations in LTP (371).

SelM and IRS-2 are both highly expressed in the hypothalamus (100,351). Absence of SelM may contribute to oxidative stress, however, other selenoproteins that have antioxidant function may be compensating. Since females have been reported to have greater levels of GPx, a known antioxidant, therefore it is plausible to expect that SelM deletion may effect males more severely than females. Even though SelM$^{-/-}$ female mice had alteration in corticosterone, thus potentially effecting HPA axis, the complexity of hormone regulation and sex differences such as estrogen, may play a role in preventing female mice from the deleterious effect on synaptic plasticity seen here in SelM$^{-/-}$ mice in this study. Altogether, these data show that the SelM$^{-/-}$ male mice have altered hippocampal synaptic plasticity. We show that SelM$^{-/-}$ male mice are deficient in hippocampal long-term potentiation (LTP), which lend mechanistic insight to the function of SelM in brain.

These findings provide novel insights on the role of SelM in maintaining proper hippocampal function, a region important in consolidating memory and learning. These studies illuminate the gender-specific role of SelM in the brain and its relevance to neurodegenerative diseases involving learning and memory. In conclusion these results have begun to help characterize and understand the phenotype observed in the SelM KO mice, and the deficits apparent from genetic deletion of SelM.

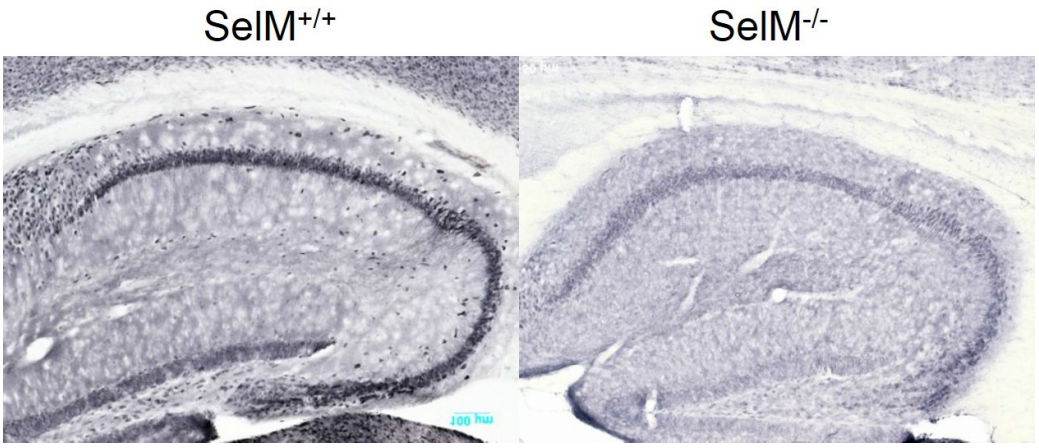


Figure 18. SelM expression in hippocampal neurons. Immunohistochemistry confirm SelM expression in hippocampal neurons and absence in SelM$^{-/-}$ mice brain sections.

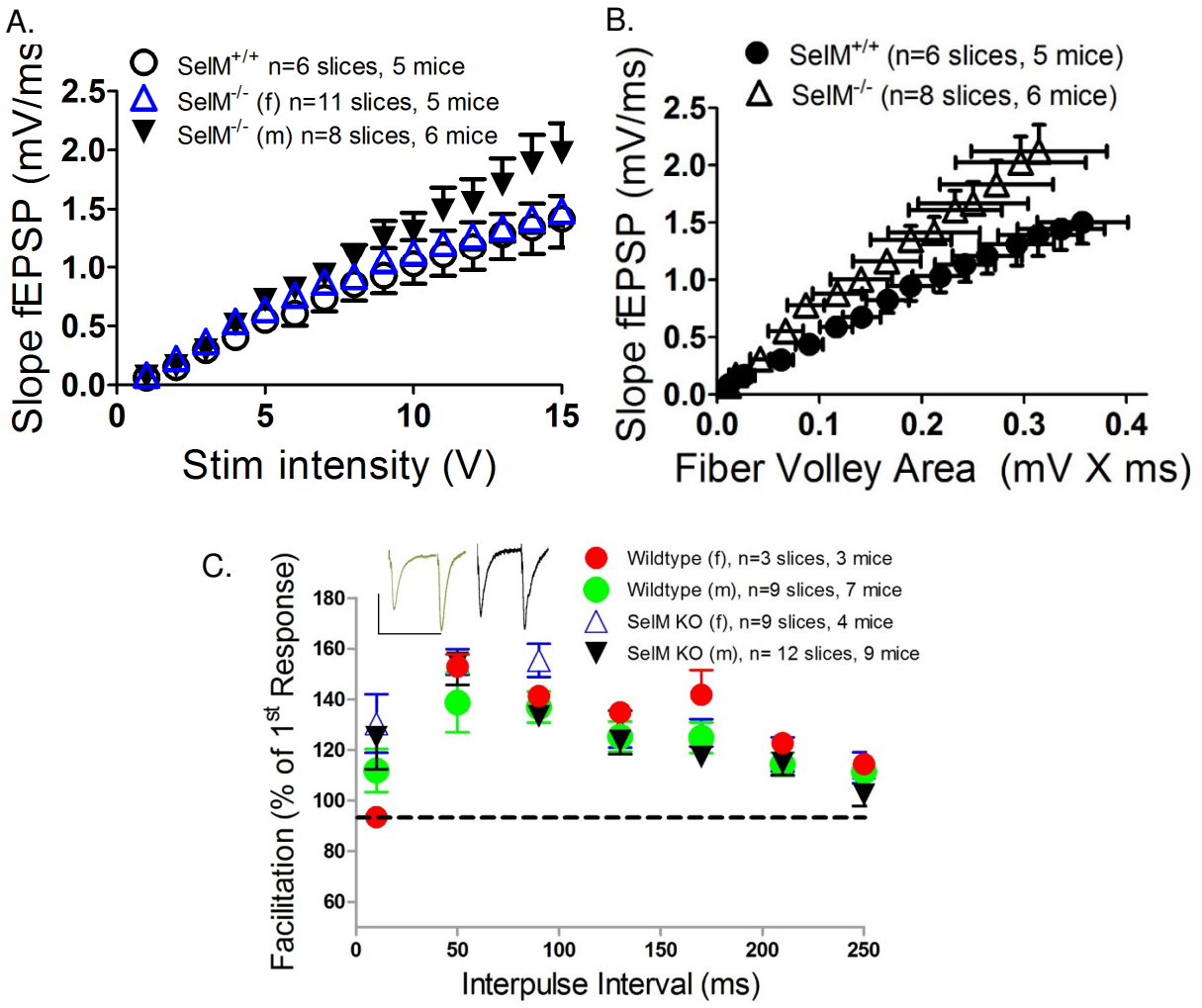


Figure 19. Deletion of SelM alters synaptic strength but not paired pulse facilitation (PPF) in SelM$^{-/-}$ male mice. (A) CA1 field excitatory postsynaptic potentials (fEPSP) in response to increasing stimulation of Schaffer collateral fibers appear increased with higher stimulus strength in SelM$^{-/-}$ male but not female mice relative to controls but are not statistically significant via Mann-Whitney test ($p>0.05$). (B) SelM$^{-/-}$ male mice have overall significantly increased fEPSP slopes relative to the area of the corresponding fiber volley compared to SelM$^{+/+}$ males. SelM deletion increased the postsynaptic response to fixed presynaptic depolarization in SelM$^{-/-}$ male hippocampus. Nonlinear regression analysis confirms that the curves are different ($p<0.0001$). (C) Absence of SelM does not change paired pulse facilitation (PPF) between genotypes or sex ($p>0.05$, two-way ANOVA).

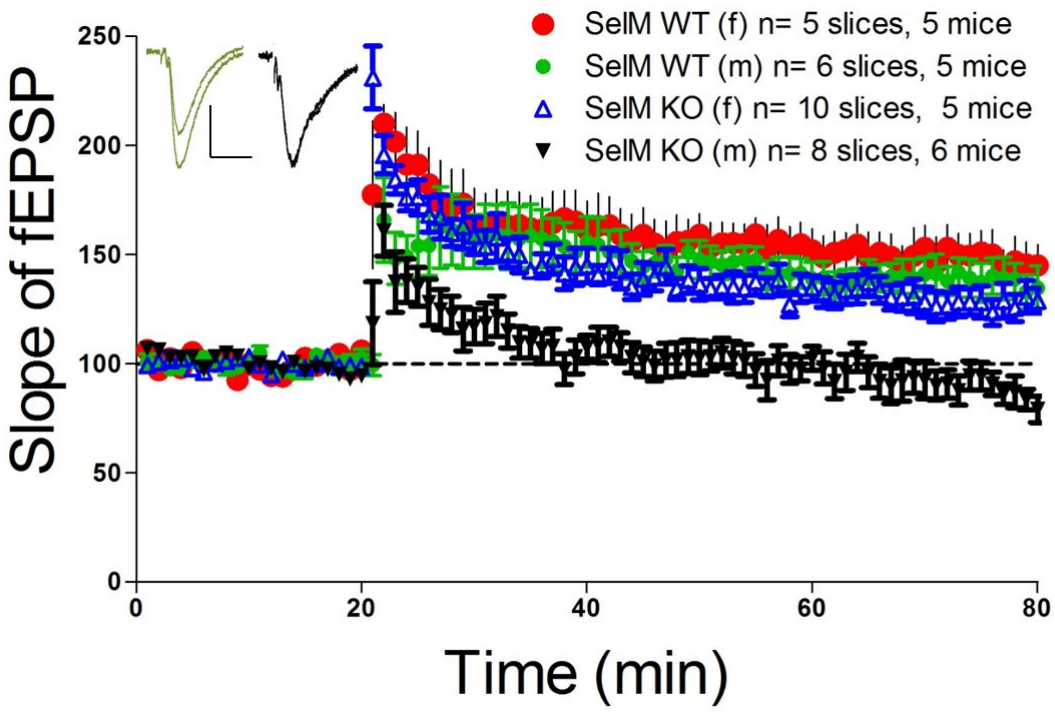


Figure 20. Deletion of SelM leads to sex-specific long-term potentiation (LTP) deficiency in SelM$^{-/-}$ male mice. SelM$^{-/-}$ male but not SelM$^{-/-}$ female mice are deficient in LTP compared to wild-type male and female mice. LTP was induced by high-frequency stimulation (HFS) (two 100 Hz trains, 20 sec apart) compared to wild-type mice ($p=0.0006$, two-way ANOVA). The fEPSPs in SelM$^{-/-}$ male mice are significantly lower than slices from wild-type animals starting from 6 min after HFS until end of recording ($p<0.001$ to 0.05, two-way ANOVA with Bonferroni posthoc tests). SelM$^{-/-}$ female mice have normal LTP compared to wild-type female counterparts. Inset: Representative trace, scale bars are 1 mV by 10 ms.

Chapter VI
CONCLUSION

Selenium and selenoproteins have been shown to be central to human health. In the last few years, we have learned a great deal more about the important roles selenoproteins. The significance of our study focused on elaborating on the specific neurological function of selenoproteins in synaptic plasticity, the basis for learning and memory formation. Increasing our knowledge of Sepp1 and SelM in synaptic plasticity will help provide mechanistic insights which will contribute towards a better understanding in how selenoproteins can be used to improve treatments and therapeutics for neurodegenerative conditions such as Alzheimer's disease.

Selenoprotein P has the critical function of transporting selenium, required for life yet its specific role and mechanism in maintaining synaptic function remains unknown. Here we attempt to examine the molecular basis of the specific role of Sepp1 and SelM in learning and memory. We present new data, in which we developed a unique mouse model restoring Sepp1 expression to forebrain neurons and demonstrating that genetic restoration of Sepp1 rescues synaptic plasticity deficits seen in Sepp-/- mice independent of Sepp1 synthesis and transport from hepatic cells.

In Chapter I, we introduce you to the wonderful world of selenium and selenoproteins. We provide an overview of the selenoprotein family and specifically discuss the importance Sepp1 and SelM in normal brain function, as well as in their role in human disease. Chapter II describes how we developed our unique genetic rescue of SEPP1 from $Sepp^{-/-}$ mice, by using the Cre System to restore the functional gene. Chapter III focuses generating a Sepp1 Conditional Brain Rescue and demonstrating that Sepp1 restricted to forebrain cells is sufficient to restore synaptic plasticity deficits seen in $Sepp^{-/-}$ mice despite absence of Sepp1 in liver to presumably transport Se to the brain. This shows that Sepp1 has other functions in the brain and restored synaptic plasticity is independent of the transport function. Chapter IV provides insights on the potential mechanism by Sepp1 functions. Chapter V. describes the new function of SelM in regulating gender-specific synaptic plasticity. Deletion of SelM results in males deficient in LTP, the molecular basis of memory formation. We discuss potential

mechanistic insights in which SelM functions to modulate gender-specific synaptic plasticity and how the metabolic dysfunction may play a role in synaptic dysfunction.

The molecular pathway of Sepp1 has not been investigated. There have been no other studies investigating whether Sepp1 modulates Dab1 upon binding to ApoER2, as when Reelin binds to ApoER2. Potentially, Sepp1 may regulate PSD-95, a scaffolding protein in the post synaptic densities (PSD) which associates with ApoER2, and is crucial for receptor clustering.

Reelin, APOE receptors, the amyloid-beta protein and cholesterol genetically or biochemically interact with each other, and have also recently all been found to function directly at the level of the synapse, where they regulate NMDA (*N*-methyl-d-aspartate) receptor activities (301). This functional convergence on a central neuronal organelle that is crucial for neurotransmission, cognition and memory throws a spotlight on APOE receptors and their potential roles in neurophysiological and pathological mechanisms (301) normal Reelin signaling requires the wild-type NFDNPVY sequence and likely the interaction of Apoer2 with Dab1 (288).

Sepp1 binds specifically to the β-propeller domain of apoER2. The extracellular region of mouse apoER2 consists of four types of protein domains which include the ligand binding repeats (LBR), the epidermal growth factor repeat (EGF-repeat), the YWTD β-propeller domain, and the O-linked sugar domain (287). Sepp1 putatively binds the extracellular region of apoER2, which consists of LBRs (287). The Sepp1 heparin binding site is located in the N-terminal domain (residues 79-86) (287,372), and heparin binding facilitates Sepp1 uptake in the rat L8 muscle cell line (287,373). In human plasma, proteases of the kallikrein family cleave Sepp1 into N-terminal and C-terminal fragments (287,374). Interestingly, the heparin-binding site in the N-terminal domain of Sepp1 is not an essential domain for apoER2 binding (287). This study found, importantly, ApoER2 binds specific isoforms of Sepp1 in the C-terminal selenocysteine-rich domain and that they bind to the β-propeller domain of apoER2 suggest that Sepp1 uptake by apoER2-mediated endocytosis is highly efficient for selenium gathering, and further, that the mechanism of Sepp1 uptake is distinct from the LBR domain-mediated uptake of other ligands (287).

In another interesting study investigating the role of ApoER2 in the testes, it was found that there are specific domains on ApoER2 that are crucial for regulating synaptic function in the brain but are not involved in the uptake of selenium in either the brain or the testes (286). Se levels were greatly reduced in both the brain and testis of Apoer2 KO mice (102,375). The results were concluded by observations that in contrast, none of the ApoEr2 intracellular domain mutants had significantly lower selenium levels in brain or testis, compared to wild type animals (286). Endocytosis in the Apoer2*[Dab-]* animals were affected by a three amino acid mutation, but it was not sufficient to decrease selenium levels in the brain or testis of these mice (286). WT animals and all of the Apoer2 ICD mutants showed vesicular Sepp1 staining at the basal lamina. There were no Sepp1 positive vesicles detected in the Apoer2 KO testes as previously reported.

These studies cited here provide further evidence to support our hypothesis that Sepp1 has a specific role in brain function including synaptic plasticity and possibly protecting against neurodegeneration which is independent of its function to transport selenoproteins and that Sepp1 may be involved in the modulation of Dab1. Further elucidating the mechanisms and diverse functions of these selenoproteins will make critical contributions to the understanding of selenoproteins and its relevance to human health to potentially utilize this knowledge to improve treatments for many diseases and pathological conditions.

We show clear evidence that both Sepp1 and SelM are critical in regulating normal synaptic function. We demonstrate that SelM deletion contribute to sexual dimorphic defects in synaptic plasticity as shown by abolished LTP responses in SelM$^{-/-}$ male mice. The sex-specific role of SelM in maintaining synaptic plasticity may be associated with its effects on metabolic regulation as previous reports that SelM$^{-/-}$ males have increased fasting insulin levels (100). Further investigation is needed to understand how SelM regulates synaptic plasticity in relationship to insulin signaling.

Sepp1 has specific role in regulating hippocampal dependent synaptic plasticity despite absence of Sepp1 in liver and is independent from transporting selenoproteins from hepatic cells. Sepp1 may regulate synaptic plasticity through the disabled-1 (Dab-1) signaling pathway similar to the actions seen upon Reelin binding to ApoER2, which

also binds Sepp1. These findings, along with evidence that Sepp1 is associated with AD pathology (91), warrant further investigation to determine which signaling pathways are involved and how Sepp1 modulates synaptic plasticity and serve as to modulate neurodegeneration.

Future

To further investigate the mechanism of Sepp1 in synaptic plasticity, using the Sepp1 Brain Rescue mouse model, we can assess the various different types of memory using electrophysiology protocols such as LTD, to help determine which mechanism Sepp1 is involved in. We should also examine Reelin protein expression levels in Sepp1 wildtype, brain rescue and KO brain tissue. To determine whether both Sepp1 and Reelin binding to ApoER2 are required for normal hippocampal synaptic plasticity, Reelin activity can be inhibited while inducing LTP. Determine whether Sepp1 Brain Rescue maintains LTP independent of endogenous Reelin by inhibiting Reelin activity (in Sepp1 Wt acute hippocampal slices) with CR-50 antibody, inhibiting Reelin multimerization. Alternatively, optogenetics could be utilized in conjunction with electrophysiology methods to inhibit Reelin or Dab1 signaling in Sepp1 mouse brain slices. A similar method was used in which optogenetics inhibited reelin signaling, thus improving the integration of grafted purkinje cells into adult cerebellum, while responses to evoked excitatory post-synaptic currents were recorded to ensure the health of the purkinje cells (376).

For further confirmation of the role of Sepp1 restricted to forebrain neurons and whether other selenoproteins are modified in this model, GPx4 protein expression in the brain can be analyzed. Additionally SelM expression can also be measured, since we now know that SelM plays a role in regulating synaptic plasticity. In adult brain mitochondrial and cytosolic GPx4 isoforms have detected in neurons of cerebral cortex, hippocampus, and cerebellum whereas glial cells were devoid of GPx4 and greater evidence points to GPx4 having a protective role in neurodegeneration (130,377). Further neurobehavioral assays such as the MWM and an elevated T-maze could be conducted to support the current data.

To elaborate on which pathways Sepp1 may be involved in, it would be worth examining the Disabled-1 pathway by initially repeating the experiment in Chapter 4 with larger parameters to increase sample size, measuring protein expression ratios of phosho-Dab1 to whole Dab1 in $Sepp^{+/+}$, $Sepp1^{-/-}$, and Sepp1 Brain Rescue. Assess expression of proteins downstream of Dab1- PI3K, PSD-95 and SFK activation. Phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB), can also be measured in Sepp1 mice as these are activated upon tyrosine phosphorylation of Dab1. In fyn-deficient neurons, upregulation of Dab1 expression and not pDab1, is consistent with a response mechanism by which the cell attempts to compensate for reduced kinase availability (304). Measuring Dab1 would provide us information about the basal levels prior to phosphorylation. In Sepp1-/- vehicle treatment for instance, we would expect that since there is significantly less pDab1, there would be an increase in Dab1.

The sexual dysmorphism effect on LTP observed in male mice upon deletion of SelM should be further investigated. It has been shown that estrogen, present in greater levels in female mice, increases Se concentration potentially protecting cells from oxidative stress (378). Comparison of various selenoprotein levels involved oxidative stress and brain function between SelM male and female mice should be conducted. Another aspect to investigate is to determine whether Se supplementation or estrogen will recover SelM LTP deficits seen in $SelM^{-/-}$ male mice.

SelM mice exhibit similar LTP deficits and behavior as both the $IRS^{-/-}$ and $NR2A^{-/-}$ mice, thus LTP deficits seen in $SelM^{-/-}$ mice may be associated with other types of learning that have not yet been examined. Studies have shown that in AD mice models, aberrant defects in NMDA-mediated calcium effects the ryanodine receptors, which are ER calcium regulators (379). We can investigate whether SelM deletion modulates NMDAr subunit expression and phosphorylation, IRS-2 and its downstream effectors such as PSD-95 and others. The role of SelM in ER store calcium regulation and protecting against oxidative stress may also contribute to synaptic plasticity dysregulation. In conjuction with the description of NR2A and IRS2 knockout mouse studies, providing further evidence that deficiency in LTP can be a result of different types of learning and memory, which may require different and more specific behavioral paradigms to determine the behavioral deficit associated with the synaptic plasticity

deficit. We can examine whether SelM$^{-/-}$ mice compared to wildtypes and also compared by gender among genotypes, have altered protein expression of NMDAR and its subunits in various brain regions such as hippocampus and hypothalamus, which can be further confirmed by immunohistochemistry.

We can also examine whether SelM deletion, effects expression of IRS-2, which is essential for insulin signaling, as SelM$^{-/-}$ mice were which was previously shown to exhibit metabolic dysfunction in a gender specific manner very similar to those seen in IRS-2$^{-/-}$ mice. Alternatively, we can examine this relationship indirectly by assessing whether SelM deletion effects IRS-2 downstream targets of the PI3K pathway and GSK-3β. Studies investigating the specific role of brain insulin signaling in neuronal functions, reported that in a brain restricted IRS expressing model, NesCreIrs2KO mice exhibited deficits in NMDA receptor-dependent synaptic plasticity in the hippocampus of mice, with a concomitant loss of metaplasticity, the modulation of synaptic plasticity by the previous activity of a synapse (369).

To further determine what type of learning and memory is effected by the synaptic plasticity deficits seen in SelM-/- male mice, different neurobehavior assays such as the elevated T-maze for a spatial non-matching-to-place test, radial maze as access whether the synaptic deficit reported in this study is associated with other types of learning, such as spatial working memory can be conducted. Furthermore, to further determine the mechanism in which SelM effects synaptic plasticity we can assess whether SelM$^{-/-}$ male mice will recover normal LTP by modify the LTP protocol as its possible their threshold for induction is higher. Potentially, SelM$^{-/-}$ LTP deficits may be restored if a stronger depolarization caused by the stronger tetanic stimulation is administered, which would activate NMDA receptor channels more efficiently, providing insight into whether the impairment of LTP is likely to be the result of an increased threshold for the LTP induction. We could futher determine if the LTP impairment is ascribed solely to the increased threshold for the LTP induction and is not due to the alteration of LTP expression mechanisms, the saturation level of LTP in SelM$^{-/-}$ male mice would be expected to be the same as that in the wild-type mice. This could be determined by saturating LTP, by repeatedly applying high-frequency stimulation until no more potentiation can be observed (324).

The findings of this study, not known to be previously reported characterize the specific function of Sepp1 and SelM in hippocampal dependent synaptic plasticity, important for learning and memory and shed light on the potential mechanisms involved. Further investigation of Sepp1 and SelM are necessary to determine the mechanisms through which Sepp1 and SelM functions to modulate synaptic plasticity functions.

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