# Sex-Specific Brain Auditory and Motor Tract Neurodegeneration and Seizure Development in Mice with a Deficiency in Micronutrient Selenium Metabolism

**A Dissertation Submitted to the Graduate Division of the University of Hawai‘i Mānoa in Partial Fulfillment of the Requirements for the Degree of**

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# Abstract

Selenium (Se) is an essential micronutrient element linked to many biological functions in human health (1). Two proteins involved in Se metabolism are Selenoprotein P (Sepp1) and Selenocysteine lyase (Scly). Previous studies in our lab showed that when both Sepp1 and Scly were disrupted, male double knockout Se-supplemented mice (MDKOSe) exhibited decreased survival, severe neurological impairment and susceptibility to audiogenic seizures (AGS) (2).

Further investigation indicates that between 70 and 80% of MDKOSe mice will develop seizure characteristics between 8 and 12 weeks of age, with about 40% requiring euthanasia prior to 10 weeks. A significant decrease in motor coordination starting around 6 weeks of age along with wobbly or abnormal gait precedes seizure development. Seizures occur within a week of weight loss commencement, accompanied by a decrease in food and water intake. Female DKOSe mice have decreased motor coordination starting at 7 weeks of age and increased percentage of inguinal and gonadal white adipose tissue fat deposits and elevated serum glutathione peroxidase 3 levels. The neurological deficit becomes severe, leading to seizure development if high selenium supplementation is removed at weaning.

Disruption of the maturation of GABAergic inhibition seems to be the main neurological effect leading to the development of AGS in MDKOSe mice. Preadolescent castration rescues the MDKOSe phenotype. It delays onset of neuromotor deficits in male Sepp1 knockout mice on a Se deficient diet and is protective of male wild type mice exposed to the neurotoxin dizocilpine maleate (MK-801). Testosterone re-administration to sham-operated MDKOSe mice appears to be beneficial but not preventative.

Together, these results indicate that there is a critical time point in which Se is essential for the prevention of neurological impairment in both female and male DKOSe mice. MDKOSe may have a greater need for Se during the course of their sexual reproductive period due to competition between the testes and the brain for Se. The specific deficits in behavioral tests and underlying GABAergic system maturation disruption could allow DKOSe mice to potentially serve as a model for the study of ictogenesis and therapeutic drug testing of novel drugs for epileptic patients.

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# Abbreviations and Symbols

| | |
|---|---|
| AGS | Audiogenic Seizures |
| AOF | Audio Open Field |
| ApoER2 | Apolipoprotein E Receptor-2 |
| Cys | Cysteine |
| Dio | Deiodinase |
| Dio2 | Type 2 Deiodinase |
| Dio3 | Type 3 Deiodinase |
| ER | Endoplasmic Reticulum |
| $GAD_{67}$ | Glutamic Acid Decarboxylase 67 |
| Gpx | Glutathione Peroxidase |
| Gpx1 | Glutathione Peroxidase 1 |
| Gpx3 | Glutathione Peroxidase 3 |
| Gpx4 | Glutathione Peroxidase 4 |
| GR | Glutathione Reductase |
| gWAT | Gonadal White Adipose Tissue |
| $H_2Se$ | Selenide |
| IC | Inferior Colliculus |
| ingWAT | Inguinal White Adipose Tissue |
| IR | Insulin Resistance |
| LDL | Low Density Lipoprotein |
| MK-801 | Dizocilpine Maleate |
| Nnt | Nicotinamide Nucleotide Transhydrogenase Gene |
| OF | Open Field |
| P | Postnatal Day |
| PNNs | Perineuronal Nets |
| $PV^+IN$ | Parvalbumin Expressing Interneurons |
| RR | Rotorod |
| rT3 | 3, 3’, 5’-Triiodothyronine |
| Scly | Selenocysteine Lyase |

| | |
|---|---|
| Se | Selenium |
| Sec | Selenocysteine |
| $SeH_2O$ | Water Supplemented with 10 μM Sodium Selenite |
| Sepp1 | Selenoprotein P |
| T2 | 3, 3’-Diiodothyronine |
| T3 | 3, 5, 3’-Triiodothyronine |
| T4 | 3, 5, 3’,5’-Tetraiodothyronine |
| Txr | Thioreodoxin |
| Txrnd | Thioredoxin Reductase |
| VLDL | Very Low Density Lipoprotein |
| XSCP | Decussation of Superior Cerebellar Peduncle |

# Abbreviations of Mouse Genotypes

| | |
|---|---|
| DKOSe | Selenoprotein P and Selenocysteine Lyase Double Knockout |
| FDKOSe | Female DKOSe Mice |
| FDKOSe-NoSeP22 | FDKOSe Mice with $SeH_2O$ removed at P22 |
| FDKOSe-NoSeP37 | FDKOSe Mice with $SeH_2O$ removed at P37 |
| FDKOSe-NoSeP42 | FDKOSe Mice with $SeH_2O$ removed at P42 |
| MDKOSe | Male DKOSe Mice |
| MDKOSe-Cast-NoSeP37 | Castrated MDKOSe Mice with $SeH_2O$ removed a P37 |
| MDKOSe-Cast P | Castrated Operated MDKOSe Mice on Placebo Pellet |
| MDKOSe-Cast T | Castrated Operated MDKOSe Mice on Testosterone Pellet |
| MDKOSe-Sham P | Sham Operated MDKOSe Mice on Placebo Pellet |
| MDKOSe-Sham T | Sham Operated MDKOSe Mice on Testosterone Pellet |
| SclyKO | Selenocysteine Lyase Knockout Mice |
| Sepp1KO | Selenoprotein P Knockout Mice |
| WT | C57BL/6, “Wild Type” Mice |
| WTj | C57BL/6JMice |
| WTn | C57BL/6N Mice |
| WTnSe | C57BL/6N Mice on $SeH_2O$ |

# Chapter 1: Selenium – the Micronutrient

## Introduction

Selenium (Se) was first discovered by Jöns Jacob Berzelius in 1817 when he identified it as the toxic element causing illness in Swedish plant workers (3). For years, it was studied due to its connection with livestock. Se toxicity in livestock leads to lameness, emaciation, and other detrimental conditions. Deficiency results in what has been called "white muscle disease" due to the white appearance of dying cell membranes in heart and skeletal muscles (3; 4). It was not until 1957 that the nutritional value of Se was recognized in the diet of rats (5). Later, in 1969, the role of Se as an essential micronutrient was established through a study of dietary deficiency in multiple rat generations (6). Se in the form of selenocysteine (Sec) was found to be a key component of glutathione peroxidase (Gpx), which is essential in the body's defenses against oxidative stress (7).

Since then, Se has been linked to many biological functions in human health including thyroid hormone synthesis and antioxidant stress defense (1). Whereas Se toxicity leads to musculoskeletal disorders (alterations in cartilage) (8), Se deficiency has been linked to clinical disorders such as Keshan disease (a cardiomyopathy) and Kashin-Beck disease (degenerative osteoarthropathy) (1) and low Se levels have been indicated in febrile seizures (9; 10; 11). Genetic mutations in genes related to Se metabolism have been shown to cause congenital muscular dystrophy (Selenoprotein N defect), progressive cerebello-cerebral atrophy (SEPSECS mutation) and retarded growth (SEC1SBP2 Syndrome) (12).

As an essential micronutrient, Se is acquired through dietary intake. Plants are the main source of Se for humans. Se accumulation in plants is dependent on soil Se (13; 14) and local climate factors such as precipitation and seasonal weather variation play key roles in Se soil distribution (7; 15). Globally, it is estimated that half a billion to one billion people are Se deficient (15; 16) and this is predicted to increase in the future due to local climate factors (17).

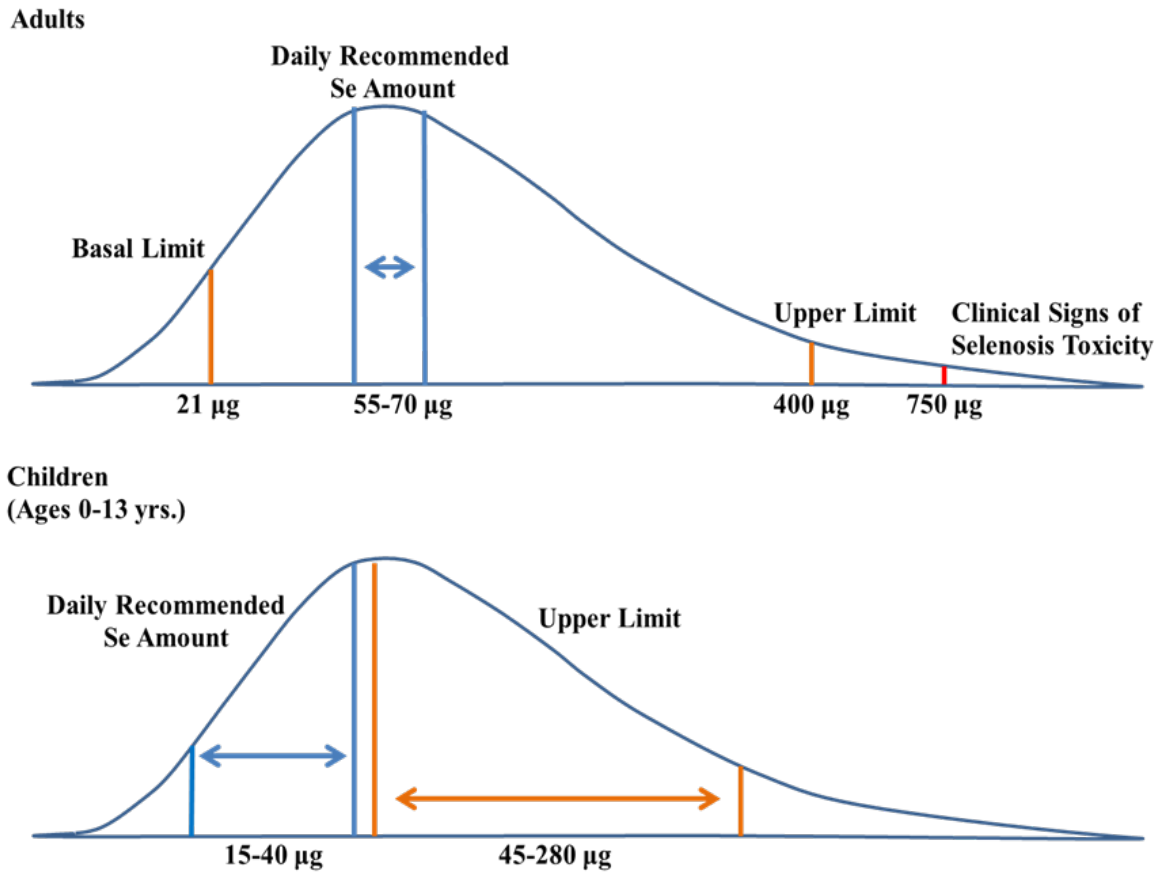



Figure 1.1: Selenium Dietary Intake Recommendations. Selenium dietary intake of adults (top) and children ages 0-13 years of age (bottom) (7; 18; 19).

The recommended dietary intake for Se varies depending on a person's stage of development. For adults, the daily recommendation is 55-70 µg/day with an upper tolerant limit of 400 µg/day. Clinical signs of toxicity are seen when intake reaches around 750 µg/day (18; 20). In children, the recommended Se intake and toxicity levels rely heavily on developmental stage with recommended intake varying between 15-40 µg and upper limits of 45-280 µg (Figure 1).

In mammals, adequate nutrient intake is a key factor in maintaining health and is important for normal growth and development. Although there are general guidelines for daily nutritional requirements, it has become evident that other factors (e.g. genetics, sex, and age) can alter an individual's nutritional needs. Malnutrition at key points of development can lead to long-term effects and chronic disease (21; 22; 23; 24). The FAO reported in 2004 that malnutrition affects about 852 million people worldwide (25). In a more recent 2015 release, it

was estimated that about 777 million people worldwide are chronically undernourished (26) and according to the 2015 WHO Global Health Estimates report, nutritional deficiencies account for about 1% of deaths worldwide (27). In children, it is estimated that malnutrition contributes to close to 5 million deaths per year and causes an additional 182 million to be physically and cognitively stunted (24). Investigation into the role of specific nutrients and the physiological pathways affecting key life stages is necessary to provide clinical targets that may help alleviate this global burden.

Table 1.1: Current FDA Recommendations for Daily Micronutrients Intake (18).

| Element | 2017 FDA Recommended Daily Amount |
|---|---|
| Iron (Fe) | 18 mg |
| Zinc (Zn) | 15 mg |
| Copper (Cu) | 2 mg |
| Manganese (Mn) | 2 mg |
| Iodine (I) | 150 µg |
| Chromium (Cr) | 120 µg |
| Molybdenum (Mo) | 75 µg |
| **Selenium (Se)** | **70 µg** |

Of the elements listed on the periodic table, nineteen elements, including Se, are considered to be essential in the human diet to provide adequate nutrition. In the US, eight are recognized as "trace" elements (micronutrients hereafter) (18) as they are required in very small amounts (Table 1.1). Micronutrients tend to have narrow biological therapeutic ranges and excess can lead to toxicity in humans, as is the case with Se.

In the last decade, there has been a shift in how malnutrition is viewed. It is evident that inadequate micronutrient intake can lead to metabolic disruption and DNA damage (28; 29; 30; 31; 32). This has been particularly important in the raising of livestock as food sources. In cattle, even though the epigenome of an animal is heritable, it can be modified by diet (29; 32) giving insight into a possible application of "personalized nutrition" in both animals and humans (21). With advancements in genomic sequencing and studies in epigenetics, it may be that in the future clinical providers can match nutritional supplementation recommendations to a person's current genomic and environmental status thereby optimizing the body's ability to remain healthy and fight physical ailments.

**Selenium absorption and metabolism**

The complete pathway of Se absorption and metabolism in the body has yet to be determined. It has been reported that Se can be obtained through dietary means in either organic forms, i.e. selenomethionine or selenocysteine, or inorganic forms, i.e. selenite, $SeO_3^{-2}$; selenide, $Se^{-2}$; selenate, $SeO_4^{-2}$; and elemental Se (8). Se can be incorporated into proteins by either specific mechanisms, i.e. selenoproteins, or non-specific mechanisms, i.e. replacement of sulfur (Figure 1.2) (1; 33; 34). Selenide ($H_2Se$) is believed to be a central component that can lead either to selenoprotein formation or Se excretion (respiratory, urine, or feces). Mechanisms of Se absorption from diet depend on the form received. Inorganic Se can be processed by simple diffusion or co-transport and exchange, whereas organic Se processing is through incorporation into the amino acids, selenocysteine or selenomethionine. Se compounds have been shown to be taken up by erythrocytes (e.g. selenite) and bind to albumin (e.g. selenide) as well as α and β globulins, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (8).

When dietary Se is deficient, the body compensates through increasing metabolites which are capable of acting as antioxidants. Metabolites involved in one-carbon metabolism leading to glutathione production are altered in Se deficiency as indicated by increased levels of serine, glycine, and threonine along with dysregulation of the methionine-homocysteine cycle (35). These can potentially cause changes in redox-methylation, lipid head group formation, and DNA methylation (35; 36; 37; 38). Se deficiency also alters phenylalanine, tyrosine, and tryptophan biosynthesis (35) which can affect levels of dopamine, norepinephrine or epinephrine, and thyroid hormones (39).

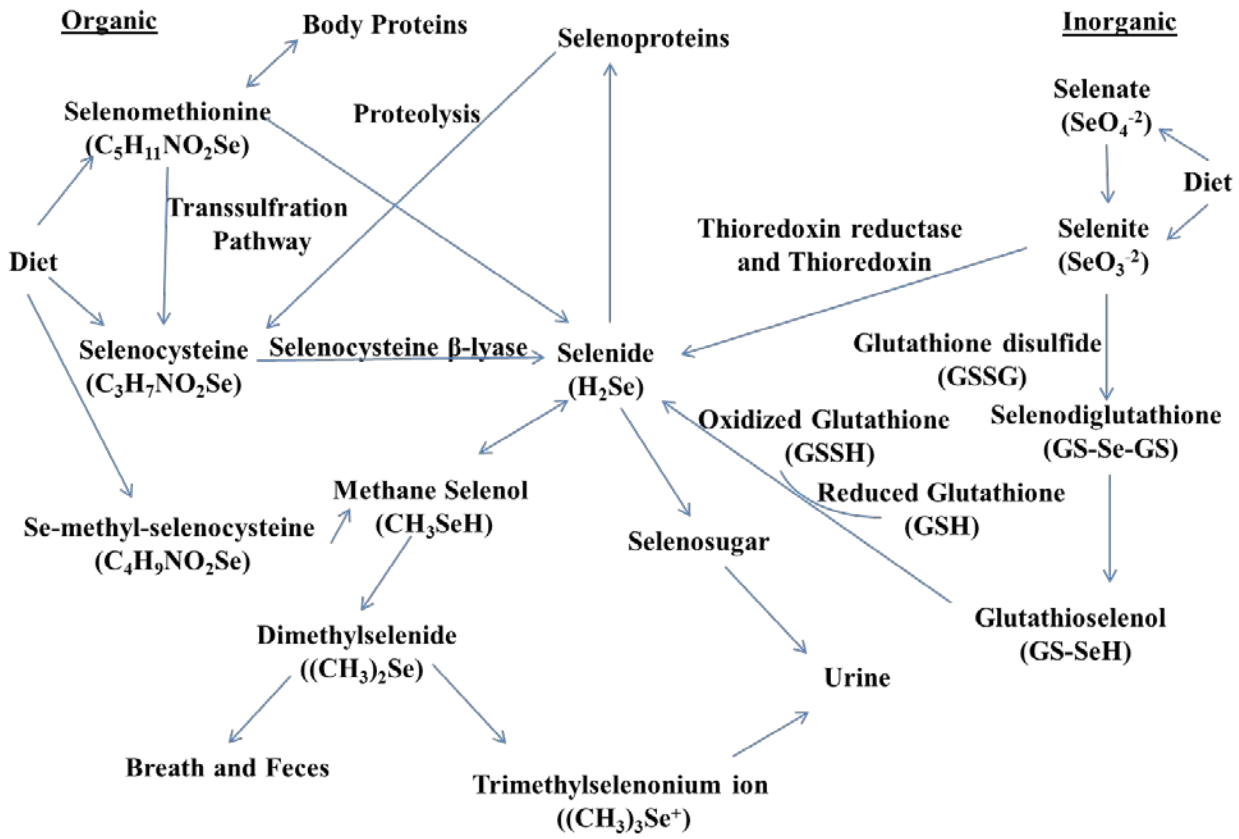



Figure 1.2: Selenium Absorption and Metabolism. Summary schematic of Se absorption and metabolism as determined from multiple studies using human tissue and rat and mouse animal models (1; 34; 40; 41).

**Selenium distribution in the body**

Se tissue distribution and concentrations vary with age and diet. With adequate dietary Se, the biological trend is that the highest concentrations are found in the kidney and liver and the lowest in the brain and lung. In humans, the muscle has the highest percentage of total body Se, ranging from 27% to 50%, whereas the liver accounts for approximately 7%, the kidney ~2-4%, and the brain ~2.5% (42; 43; 44; 45).

When rats were fed a diet deficient in Se, Se was preferentially retained in the brain, pituitary, thyroid, ovaries, adrenal glands, and testes (46; 47), with the brain given priority at the expense of other organs (47; 48; 49; 50). In the rat brain, the highest concentrations of selenium are found in the gray matter (45), including the cerebellum, and the lowest concentrations are found in the brain stem (50; 51; 52). Subcellular distribution of Se is highest in synaptosomal and mitochondrial areas (51).

**Selenium, the element**

The element Se is characterized as a metalloid resembling sulfur in chemical terms. It has six isotopes that occur naturally in both organic and inorganic forms. In humans, it is generally accepted that most or all of the biological functions of Se are manifested by its presence in the 21$^{st}$ amino acid, Sec. Sec is structurally analogous to cysteine (Cys), with Se in place of sulfur. However, Sec is not derived from Cys, but is synthesized on the carbon backbone of serine (Figure 1.3). Sec is then incorporated into 30 selenoproteins (53), encoded by one of 25 known genes (1).

Most selenoproteins incorporate Sec in their catalytic sites. Many studies have been conducted on the chemical properties that change when sulfur is replaced by Se (reviewed by Arner in 2010). Se has a higher nucleophilicity than its analog, Cys. This potentially allows it to yield faster reaction rates at physiological pH than, with higher redox potentials in non-equilibrium states (54).

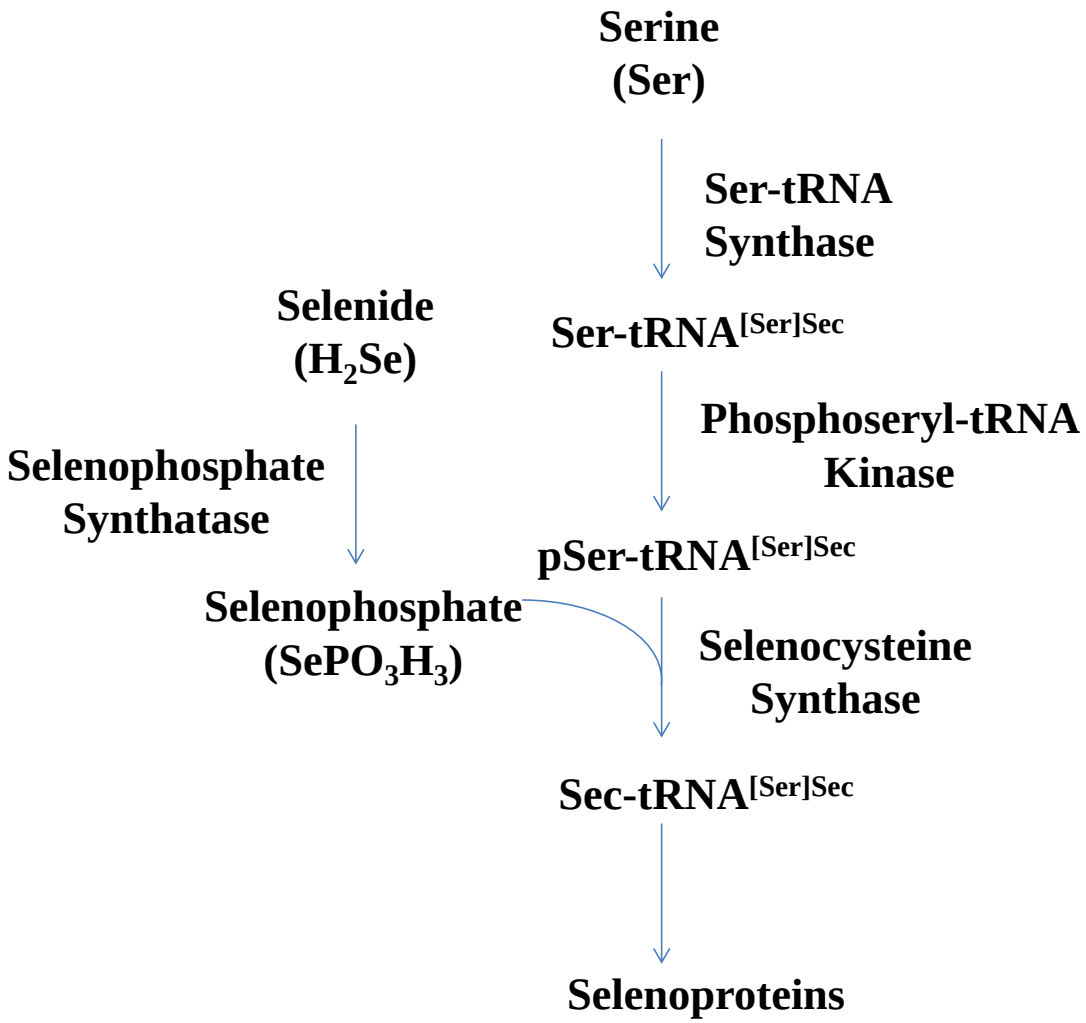



Figure 1.3: Selenocysteine (Sec) Biosynthesis and Incorporation. Serine (Ser) is aminoacylated with special $tRNA^{[Ser]Sec}$ by Ser-tRNA synthase. Ser is then phosphorylated by phosphoseryl-tRNA kinase to yield $pSer\text{-}tRNA^{[Ser]Sec}$. $pSer\text{-}tRNA^{[Ser]Sec}$ is then converted to Sec by the enzyme selenocysteine synthase which uses a selenophosphate as the Se donor producing $Sec\text{-}tRNA^{[Ser]Sec}$. Sec is then incorporated into the elongating selenoprotein. During selenoprotein synthesis, $Sec\text{-}tRNA^{[Ser]Sec}$ insertion occurs at UGA codons when directed by SECIS elements within selenoprotein mRNAs.

**Selenium and the brain**

The roles of the micronutrient Se in brain development and function have become of increasing interest. Sec is incorporated into proteins involved in growth, development, and antioxidant defense systems. These proteins include iodothyronine deiodinases (Dios), glutathione peroxidases (Gpxs) and thioredoxin reductases (Txrnds).

Iodothyronine deiodinases (Dios) catalyze activation and inactivation of thyroid hormone, which functions in many aspects of development and body homeostasis. The two predominant brain deiodinases are Dio2 and Dio3. Dio2, an endoplasmic reticulum (ER) anchored enzyme with its catalytic site exposed to the lumen, is predominately found in astrocytes, where it activates the thyroid prohormone 3, 5, 3',5',-tetraiodothyronine (T4) via conversion to the active hormone, 3, 5, 3'-triiodothyronine (T3). Knockout of Dio2 can lead to a hypothyroid state causing changes in bone and muscle, and in carbohydrate and lipid metabolism (55). Hypothyroidism during critical periods of development can lead to cognitive and behavioral dysfunction (56). It can also delay neuronal differentiation (57) and lead to an age-dependent development of ataxic gait and severe cretinism (58).

Dio3 is a plasma membrane anchored selenoprotein found in neurons and important in thyroid hormone metabolism. In the brain, it inactivates T4 and T3 by converting them to reverse 3, 3', 5'-triiodothyronine (rT3) and 3, 3'-diiodothyronine (T2), respectively. In mice, Dio3 is highly expressed during the first week after birth, then diminishes rapidly thereafter (59). Dio3 has been found highly expressed in areas of sexual differentiation in rat neonates (60). The lack of Dio3 produces a thyrotoxicosis state in mouse neonates, followed by a central hypothyroidism state at postnatal day 15, indicating its importance in thyroid axis development (59). It has been reported that there is a "hypo-responsive" period where mammals are unable to respond to stress hormones (61). This corresponds to the time when Dio3 is highly expressed. Other studies have linked transient hypothyroidism in neonatal animals to the development of audiogenic seizures in adults, with onset at puberty (62; 63).

Dio3 expression is stimulated by T3 levels. T3 has been shown to be upstream of p38, mitochondria biogenesis, AKT signaling, PI3K/ERK, and mTOR, thereby increasing metabolism and oxygen consumption (60). Dio3 inactivates T3 and therefore decreases the metabolic rate. Other pathways, including TGFB3, FGF, and HIF1α, also activate Dio3, resulting in reduced metabolic rate and oxygen consumption and increased β-oxidation (64).

The Gpxs and Txnrds have important roles in antioxidant defense. The brain has a very high energy requirement, accounting for over 70% of total body energy consumption in newborns and 20-30% in adults (65). The brain runs almost exclusively on glucose with glycogen being a short-term energy reserve. As a backup, the brain relies on ketones with fatty acid oxidation releasing twice as much energy as glycogen. Though not a requirement in adults, ketones are required during infancy, with uptake being 3 to 5 times faster in newborns (66). Ketones provide the main carbon source for brain cholesterol and long-chain fatty acids of structural lipids in development (65).

With a high metabolic rate comes the need for antioxidant defense. Se incorporation into the structure of Gpx was the key finding that confirmed its role as an essential micronutrient (7). Gpx utilizes glutathione as a co-factor in the reduction of hydrogen peroxide and phospholipid hydroperoxides, thereby neutralizing oxidative stress. Sec is incorporated into five of the eight Gpxs (67); Gpx1, Gpx2 (gastrointestinal), Gpx3 (plasma), Gpx4 and Gpx6 (olfactory). Of the five, Gpx1 is the most abundant, is found in the cytoplasm in mammalian tissues and catalyzes the reduction of hydrogen peroxide to water. Deletion of Gpx1 in mice revealed sensitivity to oxidative stress-inducing agents (68) whereas overexpression has been shown to modulate damage to long-term memory resulting from traumatic brain injury (69).

Gpx4 is essential for early development; it is the only Gpx whose deletion results in embryonic lethality (70). Gpx4 reduces phospholipid hydroperoxides, distinguishing this isoform from the other Gpxs. In the brain, Gpx4 is expressed in neurons but can be found upregulated in astrocytes in pathological states (71). Its mRNA can be detected in all stages of brain development, with regional distribution during embryogenesis (71). It has three isoforms, mitochondrial, cytosolic, and nuclear. The mitochondrial form (Gpx4-mt) appears to be important for normal brain development, as selective deletion impairs formation of the hindbrain (72). Mice with a conditional knockout of Gpx4 in neurons have decreased parvalbumin positive interneurons ($PV^{+}IN$), a subpopulation of inhibitory, GABAergic interneurons which act to synchronize neural activity, and levels are decreased in Se deficient mice that develop audiogenic seizures (2).

Gpx4 is also crucial for male fertility in mammals. The testes have a high concentration of Se (73) and male rats deprived of Se over two generations become infertile (6). At the beginning of spermatogenesis, Se in the testes begins to accumulate in high molecular weight

protein aggregates that were shown to be composed of highly cross-linked, enzymatically inactive Gpx4 (73). This is believed to be due to the essential role of Gpx4 as part of mid-piece of sperm (73; 74; 75; 76; 77) with lack thereof leading to sperm abnormalities (78) resulting in reduced fertility.

Thioredoxin reductases (Txnrds) are involved in cellular redox balance, cell signaling and DNA synthesis indirectly via their role in thioredoxin (Txn) reduction (79). Sec is incorporated into their C-terminal domains where it acts in electron transport to reduce Txn and prevents Txnrds from acting as glutathione reductase (GR) (80). Two Txnrds are ubiquitously expressed, Txnrd1 in the cytosol and Txnrd2 in the mitochondria. The third, a thioredoxin-glutaredoxin reductase, Txnrd3 resides in the testes. Similar to GR, Txnrds utilizes NADPH to reduce Txn but Txn is not their only target. Txnrds have the ability to directly reduce Txn along with other non-disulfide substrates e.g. selenite, hydrogen peroxide and lipid hydroproxides. This broad substrate specificity is what separates them from GR (81). When globally deleted, both Txnrd1 and 2 are embryonic lethal. Nervous system-specific knockout of Txnrd1 leads to growth retardation with ataxia and cerebellar hypoplasia due to decreased proliferation of granule cell precursors, whereas Txnrd2 knockout shows no obvious pathology (79). This evidence suggests Txnrd1 is more important in cerebellar development than Txnrd2.

**Proteins important for selenium metabolism**

Several reviews have been published on selenoproteins and their known functions to date (34; 45; 67; 74; 82; 83). The following chapters focus on two proteins important to Se metabolism, selenoprotein P (Sepp1), implicated in Se transport and storage, and selenocysteine lyase (Scly), involved in selenocysteine breakdown and recycling. Sepp1 is a plasma glycoprotein that consists of two domains, an N-terminal domain containing a redox motif and a C-terminal domain containing multiple Sec residues. It accounts for about 60% of the Se concentration in the plasma and is believed to be the major transporter of Se from liver to other tissues (84). Although plasma Sepp1 is secreted mostly by the liver (40), its mRNA is expressed in many tissues including kidney, heart, lung, skeletal muscle, brain, and testis (85; 86).

In Sepp1 knockout mice (Sepp1KO), both Se and Gpx activity levels are reduced in the brain and testes, indicating Sepp1 is important to both of these tissues for Se supply. Brain Se levels have been shown to be independent of plasma Se levels as long as there is local expression

of Sepp1 (40), whereas Sepp1KO combined with a Se deficient diet results in neurological dysfunction and male infertility (73; 78; 87; 88). A diet of 0.25 ppm largely prevents neurological dysfunction (87) but even with an Se adequate diet, Sepp1KO mice still exhibit deficits in contextual fear extinction, latent inhibition, and sensorimotor gating (89). A high Se diet of 1 ppm does not seem to reverse this, as mice on this diet still exhibit deficits in motor coordination and spatial learning, and altered hippocampus synaptic transmission and plasticity (90).

Apolipoprotein E receptor-2 (ApoER2) is the primary receptor for Sepp1 in the brain and testes. Similar to Sepp1KO, mice lacking ApoER2 have diminished Se levels in brain and testes with resulting infertility. They exhibit neurological dysfunction and neurodegeneration in auditory and motor areas of the brain when fed a Se-deficient diet (91; 92; 93). ApoER2 knockout mice also exhibit impaired hippocampal synaptic plasticity and cognitive deficits (94). Together, these studies indicate the importance of Sepp1 in supplying Se to the brain via ApoER2, to prevent neurological dysfunction.

Scly has been shown to selectively catalyze the breakdown of Sec into L-alanine and selenide ($H_2Se$) allowing Se to be recycled for additional selenoprotein synthesis (95). Scly, a homodimeric enzyme found in most tissues, exhibits high activity in the liver and kidneys, and is undetectable in blood and fat (96). It is necessary for balance in energy metabolism, specifically lipid and glucose metabolism, under conditions of dietary Se deficiency (83; 97). Scly knockout mice (SclyKO) fed a Se adequate diet develop normally without neurobehavioral deficits, and both male and female mice are fertile (83). When fed a Se deficient diet, fat accumulation becomes apparent as the mice age, which leads to a metabolic syndrome phenotype (97) that is more pronounced in males. SclyKO mice on an Se deficient diet also develop mild deficits in spatial learning (83).

When both Sepp1 and Scly are deleted, mice require supplementation with high Se levels to survive past weaning. They have severe neurological impairments with reduced motor coordination and increased susceptibility to audiogenic seizures (AGS), and exhibit neurodegeneration in the brainstem (2). They also show signs of GABAergic system defects with reduced density of $PV^+IN$ and $GAD_{67}$, an enzyme that converts glutamate to inhibitory neurotransmitter, GABA. This phenotype was more pronounced in the males as female mice, on the same diet, have a milder motor deficit and do not appear to develop AGS (2).

**Conclusion**

In summary, Se is a micronutrient linked to many biological functions in human health. Its biological action is through its incorporation via Sec into selenoproteins involved in growth, development and antioxidant defense systems, e.g. Gpxs, Txnrds, and Dios. When Se is deficient in the diet, it is preferably retained in the brain at the expense of other organs highlighting the importance of Se as a micronutrient for this organ. Two proteins involved in Se metabolism are Selenoprotein P (Sepp1) and Selenocysteine lyase (Scly). Sepp1 is involved in Se transport, whereas Scly catalyzes the breakdown of Sec, allowing Se to be recycled for additional selenoprotein synthesis. Though knowledge of the nutritional value of Se has expanded over the last 200 years, the biological functions and mechanisms of action of selenoproteins have yet to be fully understood. Further investigation into the roles of selenoproteins at key points of development would be beneficial in determining dietary supplementation requirements. This knowledge can then be used to optimize individualized nutrition in both humans and animals, thereby having potential application to prevent disease pathology and as a targeted therapeutic agent.

Accordingly, to further investigate the effects of disruption of Se metabolism *in vivo*, we conducted a series of experiments on male and female Sepp1 and Scly double knockout mice (DKOSe), which will be discussed in detail in subsequent chapters. We hypothesize that the neurological deficits in the DKOSe are progressive in nature in both male and female mice and that the high Se diet and/or lack of high testosterone at puberty may be protective in the case of the female mice. These studies have the potential to elucidate specific targets for further study in cases of fetal and childhood Se deficiency, and compensatory mechanisms that are distinct between males and females during development, as well as identify potential risk factors that may need to be considered in hormone replacement therapies.

# Chapter 2: Physical Features Preceding Seizure Development in Male Sepp1 and Scly Double Knockout Mice

## Introduction

Selenium (Se) is an essential trace element linked to several biological functions in human health. Through the incorporation of Se in the form of the amino acid selenocysteine (Sec), Se is thought to act mainly through selenoproteins. Selenoproteins have been shown to play an important part in the defense against oxidative stress, which has implications for the development of disease states such as neurodegeneration.

Two proteins involved in Se metabolism are Selenoprotein P (Sepp1) and Selenocysteine lyase (Scly). Sepp1 is involved in Se transport and lack of Sepp1 results in impairments in neurological function and male fertility (73; 78; 87; 88). Scly catalyzes the breakdown of Sec, allowing Se to be recycled (95). Scly is important in lipid and glucose metabolism (83; 97).

Our lab developed and characterized a novel Scly and Sepp1 double knockout (DKOSe) mouse to investigate the *in vivo* effects of disrupted Se metabolism. These mice are referred to hereafter as DKOSe, prefaced by M or F for male or female, respectively. Se supplementation of dams and pups through their drinking water was required for pups to survive past weaning. Even with Se supplementation in their drinking water, MDKOSe mice still developed neurological deterioration. They exhibited decreased survival and severe neurological impairment, including loss of motor coordination, susceptibility to audiogenic seizures (AGS) and brain stem degeneration, with GABAergic system defects in the inferior colliculus (IC) (2).

Due to the spontaneous development of seizures, MDKOSe may serve as a potential model for the study of epileptogenesis. Therefore, statistical knowledge of the incidence of seizure development as well as physical features leading up to manifestation of seizures would be beneficial references for validating intervention therapies. We hypothesize that seizure development in these mice is progressive in nature, therefore, there will be physical signs of deterioration preceding first seizure occurrence that can be monitored. These include possible changes in weight, food and water intake, general behaviors e.g. approach response, posture, coat condition, and wobbly or abnormal gait. Accordingly, we single-housed MDKOSe mice starting at five weeks of age, tested them weekly for motor coordination deterioration, and monitored

them for adverse physical signs of deterioration leading up to seizure development. Then at ten weeks of age, they underwent testing for seizures in the Audio Open Field (AOF) assay and the lengths of their aura, ictal, and postictal stages were recorded.

**Hypothesis:** Seizure development in MDKOSe mice is progressive in nature, therefore, there will be physical signs of deterioration prior to first seizure occurrence that can be monitored.

**Aim:** Identify visual and behavioral characteristics leading up to the loss of motor coordination and onset of seizure activity in MDKOSe mice and obtain the average duration of the three seizure stages, aura, ictal and postictal.

**Significance:** MDKOSe mice may serve as a potential model for the study of ictogenesis. Data obtained can be used as a reference for evidence of any changes during future investigation of mechanistic pathways and for validating possible intervention therapies.

**Materials and methods**

All animal procedures and experimental protocols have been approved by the University of Hawai'i's Institutional Animal Care and Use Committee.

*Animals:* Male and female C57BL/6J (WTj), C57BL/6N (WTn), C57BL/6N on $SeH_2O$ (WTnSe) and DKOSe mice were generated from breeders in our colony as previously described (2). Because DKOSe mice require high selenium supplementation for survival, WTnSe and DKOSe dams were maintained on a 1 ppm Se diet consisting of standard chow containing 0.25 ppm Se and water supplemented with 10 µM sodium selenite (~0.78 ppm Se) (hereafter $SeH_2O$). Pups remained on the $SeH_2O$ water after weaning. Animals were weighed and sacrificed and tissue collected thereafter at the same time of day (early evening, between 4:00-6:00 pm) to account for the 24 hour testosterone cycle. Blood was collected via cardiac puncture, centrifuged at 13,000 x g for 10 minutes and serum taken and stored at minus 80˚C until analyzed. Brains were either collected and weighed following perfusion with 4% paraformaldehyde (PFA) for histological analysis or dissected and snap frozen in liquid nitrogen and stored at minus 80˚C until use for protein, RNA, enzymatic activity or hormone analysis of specific brain regions.

*Neuromotor and Behavioral tests:* Mice were single housed 24 hours prior to behavioral testing except the group-housed mice used in preliminary studies. All tests were performed at the

same time of day to account for circadian rhythm. A select group of animals were assessed for motor coordination via Rotorod at weekly intervals from age 5 to 10 weeks. All others subjected to behavioral testing were assessed beginning at 10 weeks of age. After behavioral testing, mice were weighed, sacrificed and tissue collected as described above. Behavioral tests included the following:

- Rotorod Assay for motor coordination (ataxia). Mice were tested 4 times daily (two times in the morning and two times in the afternoon) for two consecutive days by being placed on a horizontal rod which increases rotation speed from 4 rpm to 40 rpm over a 5 minute time period. Latency to fall off the rod is averaged for the 8 trials.
- Open Field Assay for locomotion. Mice were tested by placement in an open field apparatus (50 x 50 cm) with 40 cm high opaque walls and allowed to explore for 5 minutes. Animal movement was recorded by an overhead video camera and analyzed by video tracking software (VideoMot 2, TSE Systems). Total distance traveled was recorded. Average speed was calculated as total distance traveled divided by total time.
- Audio Open Field (AOF) Assay for susceptibility to audiogenic seizures (AGS). Mice were tested for AGS susceptibility as previously described (2). Briefly, using the open field apparatus and video tracking software described above, mice were allowed to explore the open field for 2½ minutes, then were exposed to an 85 dB white noise played continuously through a loud speaker. Average speed during the pre-sound period of 2½ minutes (pre-sound) and in the initial 10 second period following the onset of white noise (post-sound) was calculated. Trials were stopped if animal seized continuously for more than 15 seconds.
- Seizure Trials. This assay was conducted similar to AOF assay above except there was a 5 minute acclimation period, followed by the 85 dB white noise being played for 15 seconds only. The total time of the aura duration (wild running), ictal duration (from the time mice fall over until they stop twitching) and postictal duration (time to regain upright on all four paws) were recorded.

*Monitoring of food and water intake:* A select group of mice were single housed starting at five weeks of age. Their body weight, food consumption and water intake were assessed two times per week. When weight started to decline, assessment was increased to daily.

*Body composition:* Following sacrifice, total body weight, brain weight and weight of fat

depots of the inguinal (ingWAT) and gonadal (gWAT) white adipose tissue were collected and recorded.

*Statistical analysis:* Statistical tests and sample number varied according to the experiment and are indicated in the text and/or figure legends. Data were analyzed and plotted using GraphPad Prism version 5 Software. All results are represented as mean ± SEM. Significance was determined by a p-value of $<0.05$.

**Results**: **C57BL/6 Genetic Background**

*C57BL/6J (WTj) vs. C57BL/6N (WTn) mice*

During the course of characterizing the DKOSe mice, we became aware of a possible complication due to genetic background. DKOSe mice were developed using selected breeding techniques (2). Briefly, SclyKO mice were bred with Sepp1 heterozygote mice. Heterozygote (Het) offspring were then mated with SclyKO mice to produce SclyKO/Sepp1Het mice. These were then crossed to produce DKOSe mice. DKOSe mice are born with less than Mendelian ratios, indicating a possible embryotic selective process (2). Since MDKOSe mice are infertile, FDKOSe are mated with SclyKO/Sepp1Het males to continue the colony.

For breeding, our lab used both WTj and WTn mice. In recent studies, it has been reported that the WTj mice have a mutation in their nicotinamide (NAD) nucleotide transhydrogenase gene (Nnt). This gene encodes NAD(P) transhydrogenase, an inner mitochondrial membrane protein which catalyzes the reduction of $NADP^+$ to NADPH. NADPH is a cofactor in ATP synthesis as well as in antioxidant defense mechanisms. WTj and WTn mice are reported to differ in metabolism and locomotor activity (98; 99; 100).

The MDKOSe mice, which have deficits in locomotion and motor coordination, do not carry the Nnt mutation. In the original study characterizing the MDKOSe mice (2), the control mice were the WTj mice on $SeH_2O$ supplementation, similar to the MDKOSe mice. It is possible that some of the differences detected were influenced by the Nnt mutation in our controls. In addition, in a study on testicular development using male Balb/c mice who were given a similar high selenium diet (1 ppm), it was found that the mice in this diet had a decrease in mRNA cFos/cjun regulation of redox status along with decreased sperm count and motility, similar to that of those on a Se deficient diet (101). It is possible that the high selenium supplementation required for our controls may cause other differences in the WTj mice.

Since both WTj and WTn mice were used in breeding, to determine if the absence of the Nnt gene had any significant effect on our previous data, we conducted the same behavioral tests that were used in the DKOSe characterization study (2) on WTj, WTn and WTn mice on $SeH_2O$ supplementation (WTnSe). At ten weeks of age, the animals were sacrificed, brain and total body weight measured and the percentage of inguinal and gonadal white adipose tissue (ingWAT, gWAT) calculated relative to the total body weight.

Sex appears to be a significant factor in motor coordination and locomotion with the females performing better than males in all three groups (Two-way ANOVA: Rotorod Sex p=0.009; Open Field Distance and Average Speed Sex p=0.0109). $SeH_2O$ supplementation has a significant effect on motor coordination with MWTnSe performing significantly better then both MWTj and MWTn, and the FWTnSe trending toward better performance than FWTj and FWTn (Figure 2.1 A). Both male and female distance traveled and average speed were similar between groups (Figure 2.1 B and C) and none showed susceptibility to AGS (Figure 2.2 A and B)

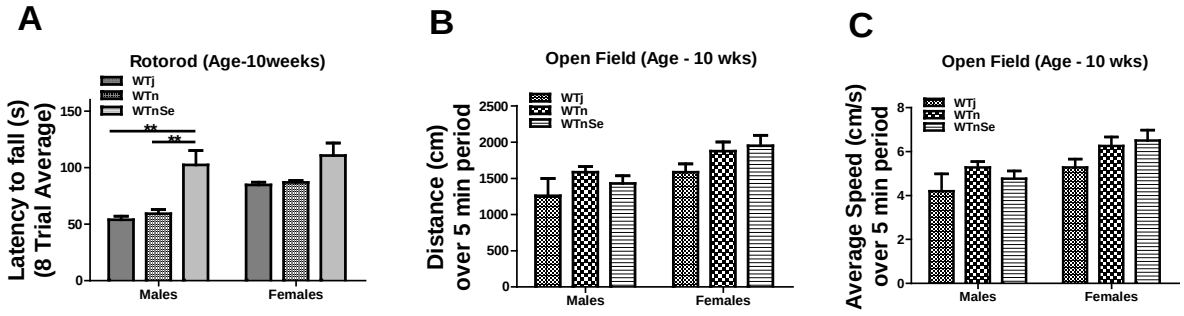



Figure 2.1: $SeH_2O$ Effect on Rotorod and Open Field Performance in WT Mice. (A) Rotorod mean latency (mean±SEM) to fall and (B) Open field distance traveled and (C) Average speed over a 5 minute period of male and female WTj, MWTn and MWTnSe mice (Rotorod: Two-way ANOVA Interaction NS, Genotype $F_{(2,44)}$=8.43, p=0.0008; Sex $F_{(1,44)}$=7.47, p=0.009; Open Field Distance: Two-way ANOVA Interaction NS, Genotype NS, Sex $F_{(1,37)}$=7.18, p=0.0109; Open Field Average Speed: Two-way ANOVA Interaction NS, Genotype NS, Sex $F_{(1,37)}$=7.17, p=0.0109; Bonferroni Post Test **p<0.01 (MWTj n=6-7, FWTj n=5-6, MWTn n=3-9; FWTn=5-8; MWTnSe and FWTnSe n=11).

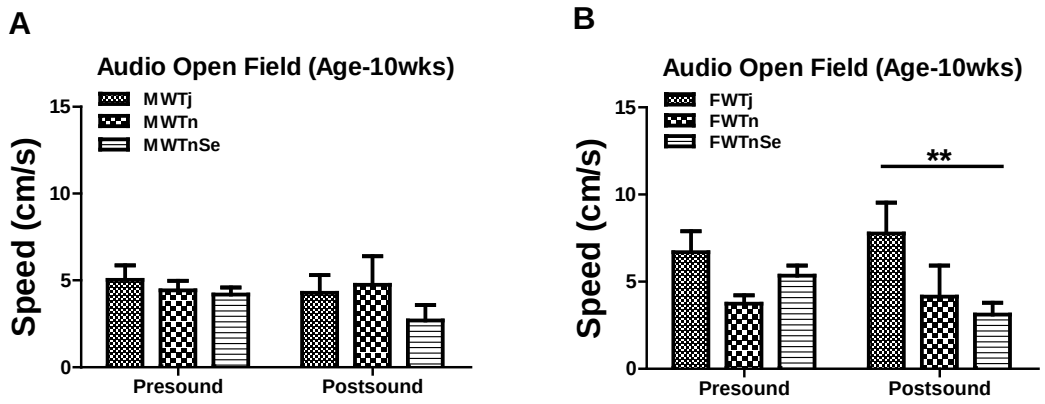



Figure 2.2: $SeH_2O$ Effect on Audiogenic Seizure Susceptibility in WT Mice. Speed prior to 85 dB white noise sounding and 10 seconds immediately after start of 85 dB white noise of (A) male and (B) female WTj, WTn, and WTnSe mice (Two-way ANOVA: (A) Interaction, Genotype and Pre verses Postsound NS; (B) Interaction and Pre verses Postsound NJ, Genotype $F_{(2,40)}=5.79$, $p=0.0062$; Bonferroni posttests **$p<0.01$; MWTj and FWTJ n=6; MWTn n=5; FWTn n=6; MWTnSe and FWTnSe n=11).

When looking at specific body weight composition, sex and genotype were significant factors contributing to differences in total body weight (Two-way ANOVA Sex $p<0.0001$, Genotype $p=0.022$), brain weight (Two-way ANOVA Sex $p=0.0141$, Genotype $p<0.0001$) and gWAT% (Two-way ANOVA Sex $p=0.0002$, Genotype $p=0.0013$). Bonferroni post hoc test revealed MWTn obtained significantly higher total body weight than both MWTj and MWTnSe. This seems to be reversed when looking at ingWAT% and gWAT%. MWTn had decreased ingWAT% and gWAT% compared to both MWTj and MWTnSe, reaching significant differences in gWAT% (Figure 2.3). For brain weight, only MWTj had significantly different brain weight compared to MWTn and MWTnSe in the males, but in females all three groups were significantly different from each other (Figure 2.3).

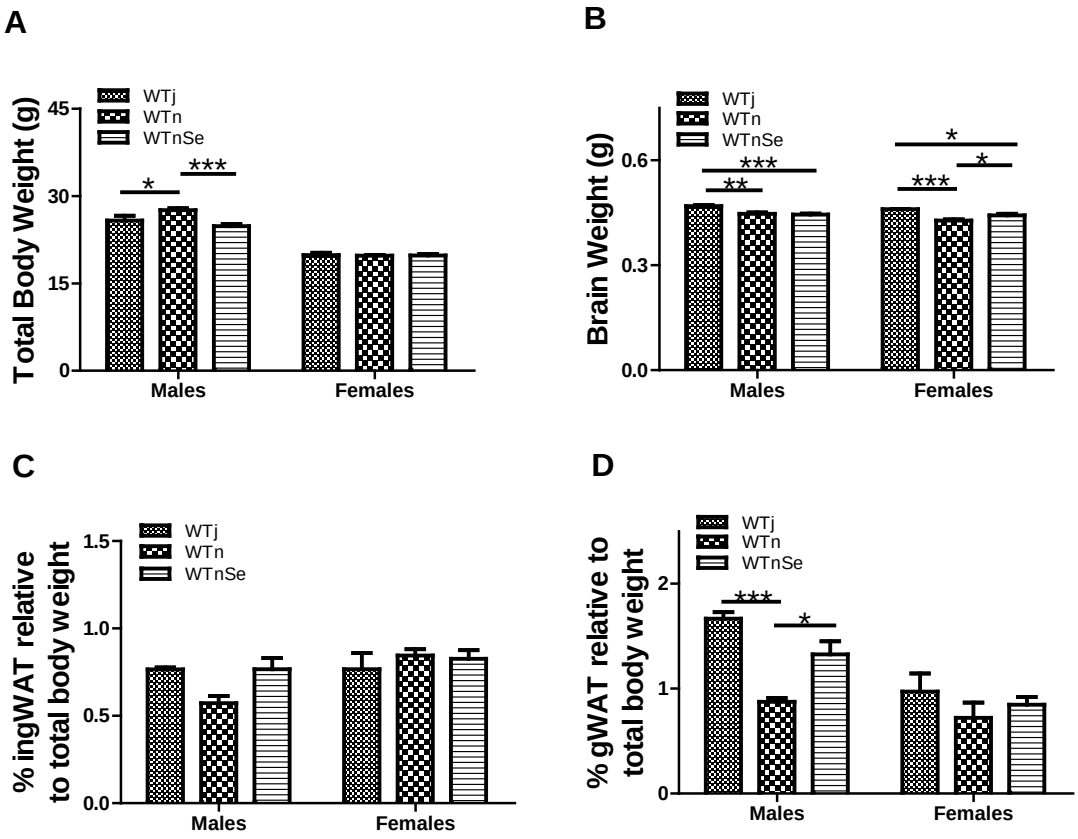



Figure 2.3: $SeH_2O$ Effect on Body Composition in WT Mice. (A) Total body weight, (B) brain weight, and (C) percentage of ingWAT and (D) gWAT of male and female WTj, WTn, and WTnSe mice. (Two-way ANOVA, Total Body Weight: interaction p= 0.0173 $F_{(2,49)}$=4.41, Genotype p=0.0222, $F_{(2,49)}$=4.12; Sex p<0.0001, $F_{(1,49)}$=251.50; Brain Weight: interaction p=0.1978, $F_{(2,48)}$=1.68, Genotype p<0.0001, $F_{(2,48)}$=0.0141, Sex p=0.0141, $F_{(1,48)}$=6.49; % ingWAT: NS; %gWAT: interaction p=0.1318, $F_{(2,46)}$=2.12, Genotype p=0.0013, $F_{(2,46)}$=7.67, Sex p=0.0002, $F_{(1,46)}$=16.47; Bonferroni Post Hoc test: *p<0.05,**p<0.01, ***p<0.001) (MWTj and FWTj n=4-7, MWTn and FWTn n=4-8, MWTnSe and FWTnSe n= 14-15).

**Results: Seizure Stage Characterization and Physical Signs for Seizure Development**

*Seizure Susceptibility*

As previously stated above, MDKOSe mice exhibit susceptibility to audiogenic seizures (AGS) starting around 8 weeks of age with few surviving past 14 weeks of age (2). The physical condition of the animals leading up to seizure and the length and duration of these seizures has

yet to be characterized. Identification of reliable visual and behavioral characteristics leading up to seizure activity in the MDKOSe mice and the average duration of their aura, ictal and postictal stages would be important parameters for identifying potential effects of any future therapeutic interventions.

Previous studies from our laboratory reported 25-40% survival at 12-14 weeks (2; 102). Analysis of two additional MDKOSe groups showed that at 8 weeks of age (Group A) about 33% of MDKOSe start to show susceptibility to seizures and at 10 weeks of age (Group B), 40% required to be euthanized prior to behavioral testing with an additional 33% developing seizure susceptibility by 10 weeks (Table 2.1). Therefore, based on these data, we estimated that about 33% to 40% of the male mice will show neurological problems with susceptibility to seizures around 8 weeks of age and additional 33% by 12 weeks, for an approximate total in the mid 60 to mid 70 percent range.

Table 2.1: MDKOSe Mice Statistics. (*Abbreviation: P = Postnatal Day)*

| MDKOSe | # of animals | Age Range | Respond to Seizure Test (Audio Open Field) | Required to be Euthanized prior to behavioral testing |
|---|---|---|---|---|
| Group A | 26 | P49-54 (7 to 8 weeks) | 33% | 0% |
| Group B | 21 | P64-86 (9 to 12 weeks) | 33% | 40% |

To further characterize the physical signs of seizure development, a select group of MDKOSe mice were single housed and monitored for behavioral deterioration. They were assessed on the Rotorod weekly starting at 5 weeks of age. Measurements were made for weight change, food and water intake, approach response, posture, coat condition, and wobbly or abnormal gait. At 10 weeks of age, mice underwent testing for seizures in the Audio Open Field (AOF) test, as previously described in Byrns et al., 2014 (2). The aura, ictal, and postictal lengths were recorded. The mice were grouped into those that had evidence of seizures prior to AOF Test (Seizure Prior AOF) and those in which had no evidence of seizures prior to the test but had a seizure when tested in the AOF test (1$^{st}$ Seizure During AOF).

*Seizure Incident and Physical Signs*

The percentage of seizure occurrence was similar to our previous results. Out of nine mice, one died spontaneously at eight weeks, 33% had evidence of seizures by nine weeks of age

and an additional 33% by ten weeks of age, making a total of 77%. For those that were observed past this age, all eventually developed AGS (n=2). Surprisingly, the average period of weight loss was the same regardless of age of seizure onset (Table 2.2). An additional group was monitored for food and water intake during their weight loss period and weight loss appears to be accompanied by an average decrease of food intake (14%) and water intake (9%) (Figure 2.4) indicating that weight loss may be used as a sign of disease progression to a deteriorating.

Table 2.2: MDKOSe Weight Loss and Seizure Onset. Average age of weight loss onset, weight loss period, period between weight loss start and seizure onset and percentage of animals of MDKOSe (n=9, *Abbreviations: P = Postnatal day, d = days*).

| MDKOSe | Ave Age Weight loss Start (P) | Ave Period of Weight Loss (d) | Ave Period between beginning of Weight Loss and Observance of 1st Seizure (d) | Ave Age Seizure Onset (P) | % of Animals |
|---|---|---|---|---|---|
| Seizure Prior to AOF | 57 | 8 | 6 | 63 | 33% |
| 1st Seizure during AOF | 61 | 8 | 9 | 70 | 33% |

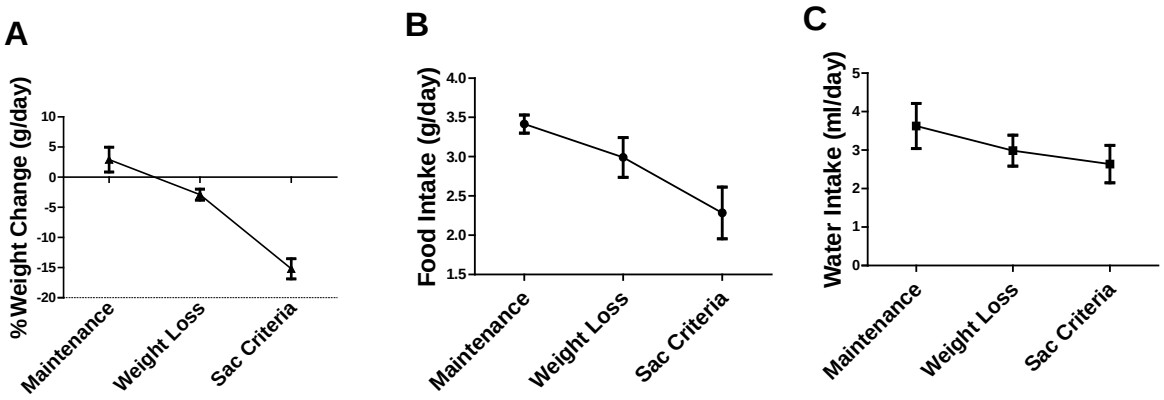



Figure 2.4: MDKOSe weight loss, food, and water intake. Percentage of weight change (A), food intake (B), and water intake (C) of MDKOSe mice which were losing weight (n=3). Weight change average was -9.0545%/day (slope of graph); Food intake slope -0.4845 or -14%/day decrease in food intake; Water intake slope -0.3169 or -9%/day decrease in water intake.

*Motor Function*

Weekly Rotorod testing revealed that MDKOSe mice have significant decreases in motor coordination compared to their wild type counterparts starting at six weeks of age (Figure 2.5). Wobbly gait was the first visual sign evident prior to the beginning of weight loss, occurring over a 2 to 5 day period (average of 4 days, data not shown). Weight loss began an average of 6 to 9 days prior to seizure development, and was more rapid in those mice that develop seizures at early ages (Table 2.2).

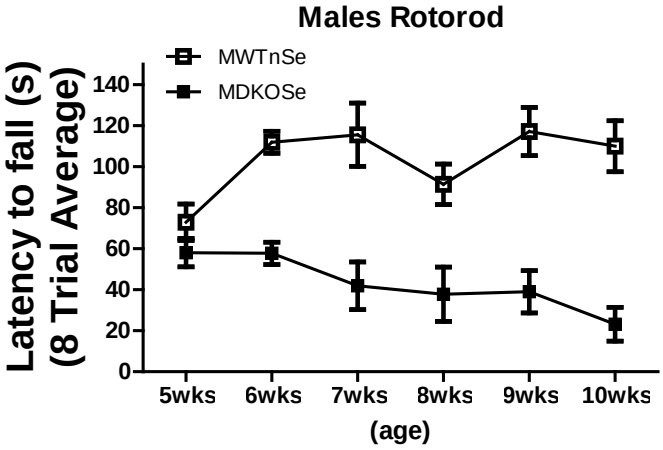



Figure 2.5: MDKOSe Weekly Rotorod Performance. Mean latency to fall of male DKOSe and WTnSe mice from five to ten weeks of age (Two-way ANOVA: Interaction $F_{(5,60)}=3.10$, $p=0.0148$; Genotype $F_{(1,60)}=100.59$, $p<0.0001$; Week NS; Bonferroni post-test

*Seizure Stages*

When the average aura, ictal and postictal lengths were compared, the differences between the two groups were mainly seen in the postictal state. Aura periods were of similar length with the ictal period being one and a half times longer in the Seizure Prior AOF group. In the postictal stage the average time it took for animals to start to move after the end of the ictal stage (1st Try) was three times longer in the Seizure Prior AOF group, and the total time to regain all 4 paws (postictal) was seventeen times longer (Figure 2.6), indicating that seizure recovery times are lengthened as the condition of the animal deteriorates.

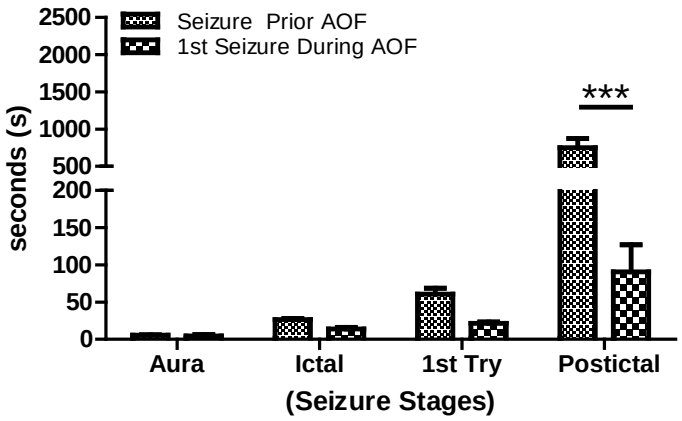



Figure 2.6: MDKOSe Duration of Seizure Stages. Average lengths of aura, ictal, 1st try, and postictal of MDKOSe mice that had seizures prior to and 1st seizure during the AOF test (Two-way ANOVA Group $F_{(1,16)}$ =31.34, $p<0.0001$; Stage $F_{(3,16)}$ =39.43, $p<0.0001$; Bonferroni posttest ***$p<0.001$, n=3)

**Discussion:**

*Genetic background of DKOSe Mice*

Under the parameters tested, the gWAT% in males and brain weight in females appear to be the only significant differences between WTj and WTn mice. The main effect of $SeH_2O$ supplementation appears to be on motor coordination, as indicated by results of the Rotorod test and the percentage of male gWAT. Though the increased Rotorod performance may appear to be a benefit, the increase in percentage of gWAT to that comparable to WTj may indicate a change in a metabolic process. The Nnt mutation has been known to affect insulin and glucose metabolism. WTj mice have been used as a model for diet-induced diabetes, with males being less glucose tolerant than females (103). Transgenic rescue of Nnt was shown to rescue insulin and glucose tolerance and β-cell function (100). However, on a low-fat diet, WTj have a slight impairment in glucose stimulated insulin secretion (104). The gWAT of WTj has been said to be limited in its storage of fat deposits and reaching this limit is a precursor to the development of metabolic syndrome disorders in MWTj mice (105). The significantly higher percentage of gWAT in WTnSe compared to WTn mice may indicate that $SeH_2O$ supplementation may be having a metabolic effect in these mice. Further observations over a longer time period along with the application of metabolic tests may be necessary to investigate this further.

The fact that no MDKOSe mice are born with the WTj mutation indicates that, with reduced Se metabolism, a functional Nnt gene may be necessary for MDKOSe embryonic survival. The Nnt gene encodes for NAD(P) transhydrogenase, an inner mitochondrial membrane protein which uses energy from the proton gradient to catalyze the reduction of $NADP^+$ to NADPH. Loss of NAD(P) transhydrogenase activity leads to a decrease in energy production and the GSH/GSSG ratio along with increased sensitivity to oxidative stress (106). It has also been reported that the enzyme may have a role in the modification of another antioxidant, the mitochondrial superoxide dismutase (mtSOD). The absence of both NAD(P) transhydrogenase and mtSOD leads to fetal dilated cardiomyopathy and embryonic death in WTj mice (106).

Se is an important component of proteins that have antioxidant activities, e.g. Txnrd. Deletion of either Txnrd1 or Txnrd2, ubiquitously expressed selenoproteins located in the cytosol and mitochondria, respectively, is embryonic lethal. Txnrd 2 deletion lethality is due to a disruption in cardiac development (79). It is possible that similar to mtSOD deletion in WTj

mice, the ability of the body to maintain redox balance is severely compromised under the extra metabolic strain of Nnt loss in combination with the reduced Se availability for selenoprotein synthesis leading to embryonic lethality. Studies on selenoproteins and their effects on embryonic development would be needed to investigate this possibility.

*Physical features preceding seizure development in MDKOSe mice*

MDKOSe spontaneously develop seizures starting around 8 weeks of age. Knowledge of the physical features leading up to manifestation of seizures may serve as a reference for validating intervention therapies. Accordingly, we carried out studies to further characterize objective signs of seizure development.

Using our previous data, we estimated that approximately 75% of MDKOSe mice that would have susceptibility to seizures between 8 and 12 weeks of age. As predicted, the percentage of mice that developed seizures in this study fell close to this estimation, being 77%. Loss of body weight appears to be a reliable indicator of when seizures would start to occur, commencing approximately a week before seizure development. This approximation was limited to our ability to observe 1$^{st}$ seizure occurrence through daily checks by animal care staff and additional observations made by the researcher at least 3 times a week. This leaves a potential 24 hour gap in time in which the animals are not observed. A 24 hour monitoring system would eliminate this and is recommended for monitoring how often seizures occur once they begin.

Weight loss was accompanied by a decrease in food and $SeH_2O$ intake which still leaves the question as to whether this is a compounding factor. Decreased food and $SeH_2O$ intake leads to further decrease of Se intake and may thereby accelerate seizure progression. It is possible that forced food and $SeH_2O$ intake could prevent weight loss but not prevent seizure development as, in the animals studied beyond 10 weeks of age, all mice eventually developed seizures. This suggests that seizure development is connected to an aging process in which each mouse is affected differently e.g. some by 8 weeks, others by 10 weeks, and still others much older.

Se deficiency has the potential to cause epigenetic changes in metabolites (35). Alterations in the levels of different metabolites involved in one-carbon metabolism have downstream effects on methylation and hormone biosynthesis which can cause a variety of

developmental effects (35; 36; 37; 38; 39). Investigations into the subtle differences that delay seizure onset in one mouse verses another may help reveal key components which determine seizure development, and reveal potential targets for therapeutic intervention.

**Conclusion**

In this study, 77% of MDKOSe developed seizure characteristics by 10 weeks of age. Decreased motor coordination started around 6 weeks of age along with wobbly or abnormal gait which preceded weight loss. Seizure development occurred within a week after weight loss commencement, which was accompanied by a decrease in food and water intake.

Based on these results, we estimate that 70-80% of MDKOSe mice will develop the seizure phenotype. This will start with loss of motor coordination along with a wobbly or abnormal gait. Weight loss will begin prior to first seizure occurrence with postictal duration increasing as the mice continue to have seizures. Using the excessive weight loss criteria, about 40% of the animals will require euthanasia prior to 10 weeks of age.

# Chapter 3: Effects of High Se Supplementation on Neuromotor Deficit in Female Sepp1 and Scly Double Knockout Mice

## Introduction

Se metabolism has been shown to differ between the sexes, with females being able to retain Se better in all tissues except the brain and reproductive organs (76; 107). Single gene knockout of Sepp1 or Scly resulted in detrimental neurological or metabolic phenotypes that were more pronounced in males. Thus, studies of these mouse models focused on males, with females only assessed in preliminary initial studies.

In previous studies published by our laboratory on Sepp1 and Scly Double Knockout (DKOSe) mice, we reported that when both Sepp1 and Scly genes were disrupted, male DKOSe (MDKOSe) mice exhibited decreased survival and severe neurological impairment, including loss of motor coordination, susceptibility to audiogenic seizures (AGS) and degeneration in brain nuclei and tracts involved in auditory and motor pathways, with GABAergic system defects in the inferior colliculus (IC) (2). Female DKOSe (FDKOSe) mice exhibit a milder phenotype so they were not examined in detail at the time of MDKOSe study.

Although the FDKOSe mouse phenotype does not appear to be as severe as that of the MDKOSe, further characterization revealed FDKOSe mice do display significantly less motor coordination than their wild type counterparts, C57BL/6N (FWTnSe), as tested by Rotorod assay at 10 weeks of age (Figure 3.1 A). Total body weight of FDKOSe was also significantly greater than FWTnSe (Figure 3.1 B). These results suggest a milder neurological phenotype with a metabolic component in FDKOSe mice. Therefore, we hypothesize that, like the MDKOSe mice, FDKOSe mice neuromotor deficit is progressive in nature and that the high Se supplementation may be protective in the female mice. To further investigate the FDKOSe neuromotor and/or metabolic phenotype, we assessed their neuromotor function by performance on a Rotorod at weekly intervals from ages 5 to 10 weeks and measured their body fat composition and fasting insulin levels at 10 weeks of age. For a subgroup of FDKOSe mice, we removed the high Se supplementation at different ages and conducted the same behavioral test previously used to characterize the MDKOSe mice.

**Hypothesis:** Similar to the MDKOSe mice, FDKOSe mice neuromotor deficit is progressive in nature and the high Se supplementation may be protective in the females.

**Aim:** Investigate the possible neuromotor deficit and metabolic phenotype in FDKOSe and determine what role the high Se supplementation may play on the female phenotype.

**Significance:** Reveal possible compensatory mechanisms that are distinct between males and females during development.

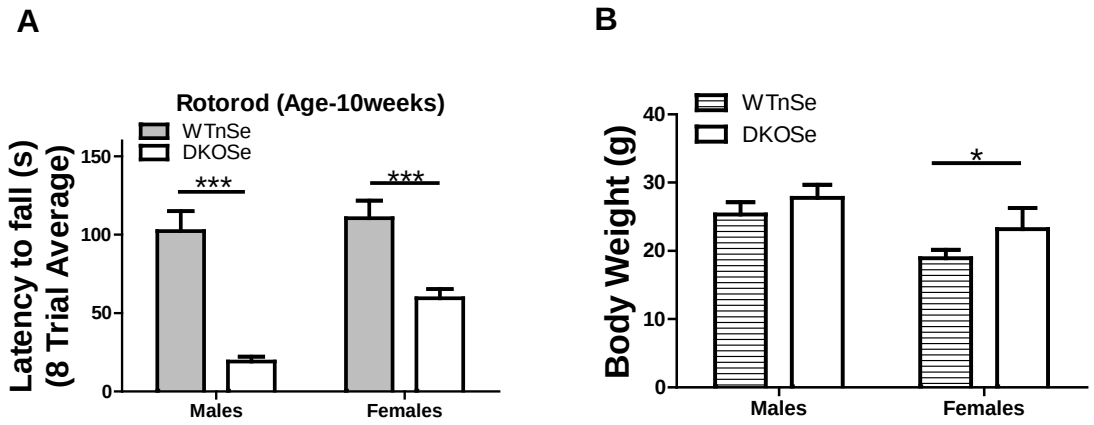



Figure 3.1: Rotorod Performance and Body Weight on Male and Female WTnSe and DKOSe Mice. (A) Mean latency to fall and (B) Total body weight of male and female WTnSe and DKOSe mice at 10 weeks of age (Two-way ANOVA: Rotorod $p<0.0001$, Genotype $F_{(1,40)}=53.63$ $p<0.0001$, Sex $F_{(1,40)}=7.05$ $p=0.0113$, n=11 all groups; Two-way ANOVA: Body Weight Genotype $F_{(1,16)}=12.40$ $p=0.0028$, Sex $F_{(1,16)}=33.87$ $p<0.0001$, n=5 all groups; Bonferroni *$p<0.05$, ***$p<0.001$).

**Materials and methods**

*Animals:* Mice were generated, maintained, and samples collected as previously described in Chapter 2.

*Neuromotor and Behavioral tests:* All analyses were conducted as described in Chapter 2.

*Body composition:* Tissue weights were collected and recorded as described in Chapter 2.

*Fasting serum insulin levels:* Animals were fasted for 5 hours then sacrificed and blood was collected at the same time of day, between 5:30-6:30 pm. It was then centrifuged at 13,000 x g for 10 minutes and the serum collected and stored at minus 80˚C until assayed for insulin levels. Insulin levels were measured using ALPCO mouse ultrasensitive insulin ELISA kit (cat#80-INSMSU-E01) pursuant to the manufactures instructions.

*Histology and immunohistochemistry*: Tissue was collected and processed as previously described (102). Briefly, mice were deeply anesthetized using 1.2% Avertin and perfused intracardially with 0.1 M sodium phosphate buffer (PB) followed by 4% PFA in PB. Brains were removed, stored in 4% PFA for 24 hours, then immersed sequentially in 10%, 20%, and 30% sucrose in PB (24 hours each solution). Brains were cut into 40 µm coronal sections. To assay for reactive gliosis, sections were incubated with primary antibody-GFAP (Dako #Z0334). GFAP was visualized using diaminobenzidine tetrahydrochloride (DAB) immunohistochemistry, sections were treated with 0.3% $H_2O_2$, blocked, and incubated in primary antibody overnight at 4°C. The sections were then probed with the appropriate biotinylated secondary antibody followed by incubation in avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector Labs # PK6100) and visualized by peroxidase detection using DAB HRP Peroxidase Substrate Kit (Vector Labs # SK4100). Sections were mounted, dehydrated with graded solutions of ethanol followed by xylene, and cover slipped.

*Silver staining:* Silver staining was performed using the FD Neurosilver Kit II (FD Neurotechnologies # PK301) according to the manufacturer's instructions.

*Protein extraction and immunoblotting:* Mice were euthanized by $CO_2$ inhalation and brains were quickly removed. Brains were cut in half along the longitudinal fissure, with one hemisphere being snap-frozen in liquid nitrogen for use as a whole-brain sample. The remaining hemisphere was dissected and the cerebellum, brainstem, and hippocampus snap-frozen in liquid nitrogen for later analysis. Frozen tissues were lysed by sonication in CelLytic MT Cell Lysis Reagent (Sigma-Aldrich # C3228) containing protease inhibitors (Calbiochem # 539134) and centrifuged at 14,000 x *g* for 10 minutes at 4°C. Supernatants were collected, and the protein concentrations were measured using Nanodrop NP-1000 spectrophotometer. For Western blotting, 40 µg samples of total protein were separated on 4%–20% SDS-PAGE gradient gels (Bio-Rad # 567-1094), transferred to Immobilon-FL PVDF membranes (VWR, 28298-022), and probed for 2 hours at room temperature with specific primary antibodies. Membranes were then

incubated in the dark with secondary antibodies coupled to infrared fluorophores (LI-COR Biosciences). Blots were imaged and analyzed using an Odyssey infrared imager (LI-COR Biosciences) together with Image Studio Version 4.0. Relative protein levels were determined by dividing the optical density of the band representing the protein of interest by that of loading controls, α-tubulin or β-actin. For determining the Gpx3 levels in the serum, Ponseau S in 1% (v/v) acetic acid was used to visualize protein bands for normalization. Blots were then washed 5 minutes in 5% acetic acid (v/v) followed by two 5 minute washes in $dH_2O$, then blocked and probed with primary and secondary antibodies as stated above. To simplify for comparative purposes, the mean normalized optical density for each protein was reported as a ratio to female wildtype (FWT) normalization.

*Antibodies:* The primary antibodies used for immunohistochemistry were as follows: rabbit anti-GFAP (1:2000; DAKO Z0334). The primary antibodies used for Western blotting were as follows: mouse anti-glutathione peroxidase 3 (1:500; LabFrontier LF-MA0114), rabbit anti-type 3 deiodinase (1:1000; Novus Biologicals NBP1-05767B), and mouse anti-α-tubulin (1:5000; Novus Biologicals NB100-690)

*Gpx activity assay:* Soluble proteins were extracted as described above. Samples were diluted to 4 μg/μl in Gpx buffer consisting of 50 mM phosphate buffer + 5mM EDTA, pH=7.4. Then 25 μl of sample were loaded into a 96 well plate and incubated at 37˚C for 3 minutes in 45 μl of master mix: 12.5 μl of 30 mM glutathione (Sigma CAS 70-18-8), 12.5 μl of 30 μg/ml glutathione reductase (Sigma 9001-48-3), and 20 μl of 1mM NADPH (Sigma 2646-71-1)). Five μl of 6 mM tert-tutyl hydroperoxide solution was then added to each sample and the plate was read by a Spectramax M3 plate reader (Molecular Devices) at 340 nm for 10 minutes. Using Softmax Pro 6.2.1 software, the Gpx activity was measured values determined as the reduction rate of *tert*-butyl hydroperoxide catalyzed by the samples upon the oxidation of glutathione and reduced NADPH. A unit of activity was defined as the consumption of 1 μmol of NADPH per minute, calculated from the equation ($V$max x $Vt/Vs$)/(0.0062 x D), using 0.0062 $\mu M^{-1} cm^{-1}$ as the extinction coefficient for NADPH at 340 nm.

*Quantification:* The optical density of GFAP immune reactivity and silver staining in the IC and XSCP was quantified at the same coronal levels, -5.02 and -4.48 mm respectively. Brightfield images (5 x objective) were captured with a digital camera mounted on a Zeiss microscope (Axioskop2), imported into ImageJ analysis software, and converted to black-and-

white images. Contours were drawn around the area of interest and the adjacent area (as background control). Mean optical density was determined as the difference between the area of interest and the background control contour.

*Statistical analysis:* All were conducted as described in Chapter 2.

**Results**

*Comparison of physical features and motor performance for male and female DKOSe mice on Se supplementation*

Weekly Rotorod averages showed that FDKOSe mice do indeed exhibit a milder neurological deficit, with "latency to fall" times that were between those of FWTnSe and MDKOSe mice (Figure 3.2). FDKOSe mice exhibit a decrease in motor coordination starting at 7 weeks of age compared to their FWTnSe counterparts. In body composition, there were no significant differences in brain weight (Figure 3.3 B). The FDKOSe mice had significantly higher total body weight (Figure 3.3 A) and percentage of inguinal (%ingWAT) and gonadal (%gWAT) white adipose tissue (Figure 3.3 C & D) when compared to their FWTnSe counterparts, but their fasting insulin levels were not significantly different when compared to controls (Figure 3.4). These results indicate that even with $SeH_2O$ supplementation, FDKOSe mice develop a neurological deficit.

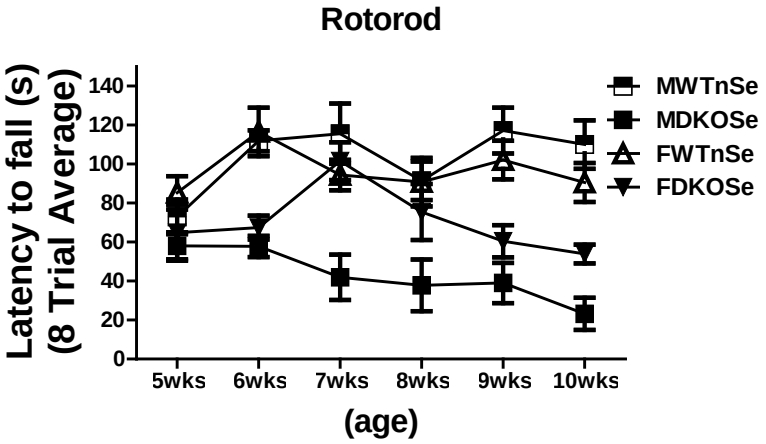



Figure 3.2: Weekly Rotorod Performance of Male and Female WTnSe and DKOSe Mice. Mean latency to fall from 5 to 10 weeks of age of male and female WTnSe and DKOSe (Two-way ANOVA: Interaction $F_{(15,120)}$=1.85 p=0.0355, Genotype $F_{(3,120)}$=42.17 p<0.0001, Week $F_{(5,120)}$=2.74 p=0.5071; Bonferroni: MWTnSe v MDKOSe 6wk-10wk p<0.01, FWTnSe v MDKOSe 6wk-10wk p<0.01; MWTnSe v FDKOSe 6, 9, 10 wk p<0.05; FWTnSe v FDKOSe 6, 9 wk<0.05; FDKOSe v MDKOSe 7wk <0.001; n=6 all groups)

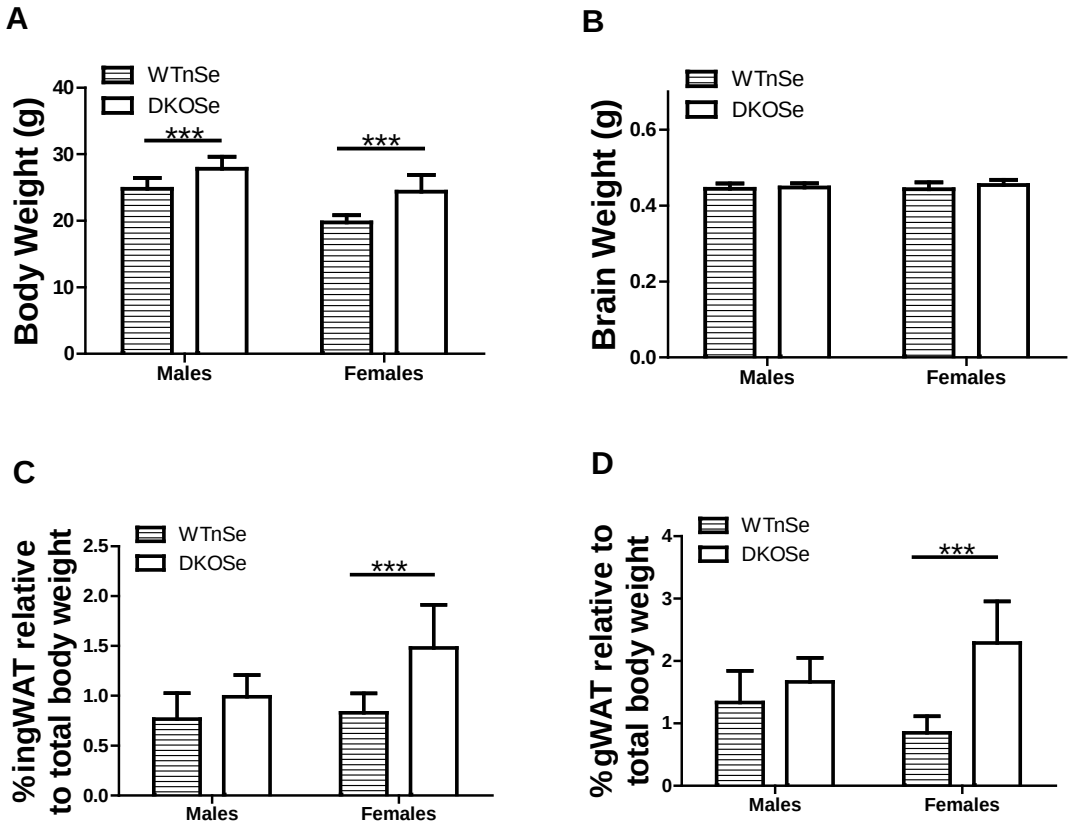



Figure 3.3: Body Composition in Male and Female WTnSe and DKOSe Mice. Body (A), brain (B) and percentage of ingWAT (C) and gWAT (D) of male and female WTnSe and DKOSe mice at 10 weeks of age (Two-way ANOVA: Body weight, genotype $F_{(1,52)}$=60.68 p=<0.0001, Sex $F_{(1,52)}$=75.15 p<0.0001; Brain NS; IngWAT Interaction $F_{(1,52)}$=7.54 p=0.0083, Genotype $F_{(1,52)}$=31.51 p<0.0001, Sex $F_{(1,52)}$=12.64 p=0.0008; and gWAT Interaction $F_{(1,52)}$=18.64 p<0.0001, Genotype $F_{(1,52)}$=47.51 p<0.0001 Bonferroni ***p<0.001, n=14 all groups).

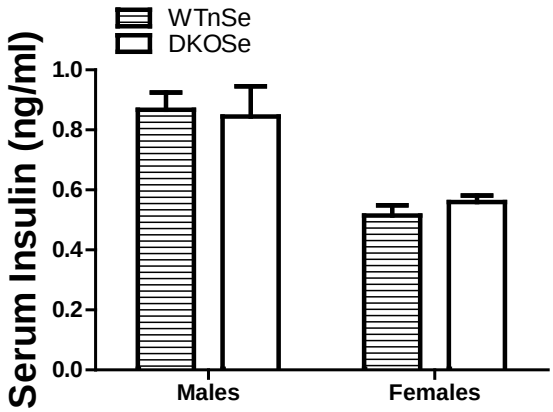



Figure 3.4: Five Hour Fasting Serum Insulin Levels in Male and Female WTnSe and DKOSe Mice. Serum insulin levels of male and female DKOSe and WTnSe mice. (Two-way ANOVA Sex $F_{(1,35)}$=19.15 $p<0.0001$; Bonferroni NS; WTnSe n=3, DKOSe n=4; triplicate)

*Impact of timing of $SeH_2O$ removal on motor performance for FDKOSe mice*

A Se deficient diet appears to aggravate the phenotype in males of both the Sepp1KO and SclyKO. To date, the FDKOSe tested were on a high Se supplemented diet through their drinking water as described in the previous chapter ($SeH_2O$). In order to determine if their motor developmental was related to Se supplementation, we removed the $SeH_2O$ supplementation from FDKOSe at different ages, postnatal day (P) 22, 37 or 42, and tested for motor coordination and susceptibility to seizure development.

Rotorod performance of FDKOSe-NoSe P42, P37 or P22 groups that survived to P70 showed a trend of decreasing latency to fall times when compared to FDKOSe mice maintained on $SeH_2O$ through P70 (Figure 3.5). Surprisingly, it was found that the performance of the FDKO-NoSeP22 that survived to P70 did not change from P34 to P83. The mice that did poorly on P34 did equally poorly on P83 (data not shown) indicating that motor deficits were acquired at an early time point prior to P70.

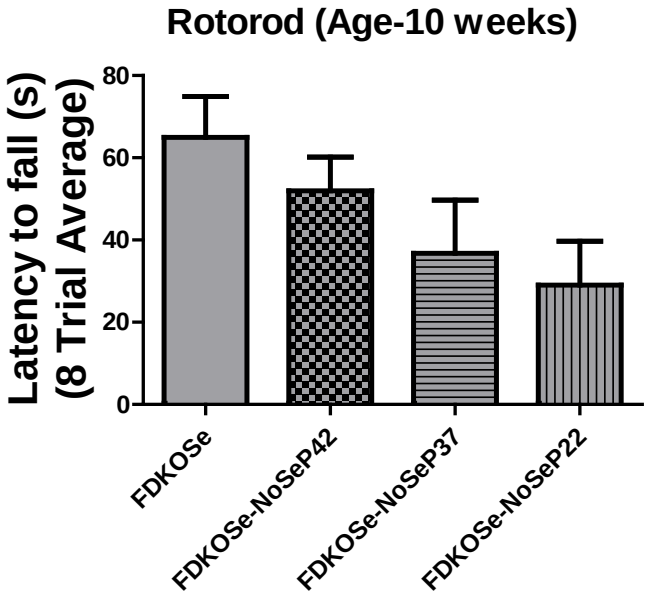



Figure 3.5: Effect of $SeH_2O$ Withdrawal Age on Rotorod Performance in FDKOSe Mice. Latency to fall of FDKOSe, FDKOSe-NoSeP42, P37 and P22 mice at 10 weeks of age (n=4 all groups except P37 n=3)

Further characterization of FDKOSe mice maintained on $SeH_2O$ through P22, P37 and P70 revealed that removal of $SeH_2O$ at P22 produced a more detrimental outcome as compared to Se removal at the later postnatal dates, resulting in motor performance approaching what we originally observed in MDKOSe mice maintained on $SeH_2O$ throughout. In the FDKOSe-NoSeP22 mice, 83% exhibited motor deficit and/or susceptibility to seizures by ten weeks of age (Table 3.1). They had significantly reduced latency to fall times compared to all other groups tested (Figure 3.6 A). Assessment of locomotion and exploratory behavior in the open field assay showed FDKOSe-NoSeP22 mice traveled significantly less (Figure 3.6 B) over a 5 minute period of time and at significantly slower speeds (Figure 3.6 C). In the audiogenic seizure assay, FDKOSe-NoSeP22 mice exhibited wild running in response to an 85 dB white noise (Figure 3.6 D).

When we looked at body composition, FDKOSe-NoSeP22 mice had significantly lower total body weight compared to FDKOSe mice with ingWAT% trending lower than FDKOSe. Their gWAT% that was intermediate between FDKOSe and FWTnSe mice with significantly

higher gWAT% compared to FWTnSe yet significantly lower gWAT% than the FDKOSe mice (Figure 3.7). Removal of $SeH_2O$ at P37 resulted in FDKOSe-NoSeP37 mice having significantly lower gWAT% compared to FDKOSe mice but no differences in the other tissue weighed (Figure 3.7).

Table 3.1: FDKOSe Motor Deficit and/or Seizures Development Percentages by $SeH_2O$ Removal Age.

| Age to which $SeH_2O$ was continued | % of mice that exhibited motor deficits and/or seizures by P70 | % of mice required to be euthanized prior to P70 |
|---|---|---|
| P22 | 83% | 28% |
| P37 | 29% | 0% |
| P70 | 0% | 0% |

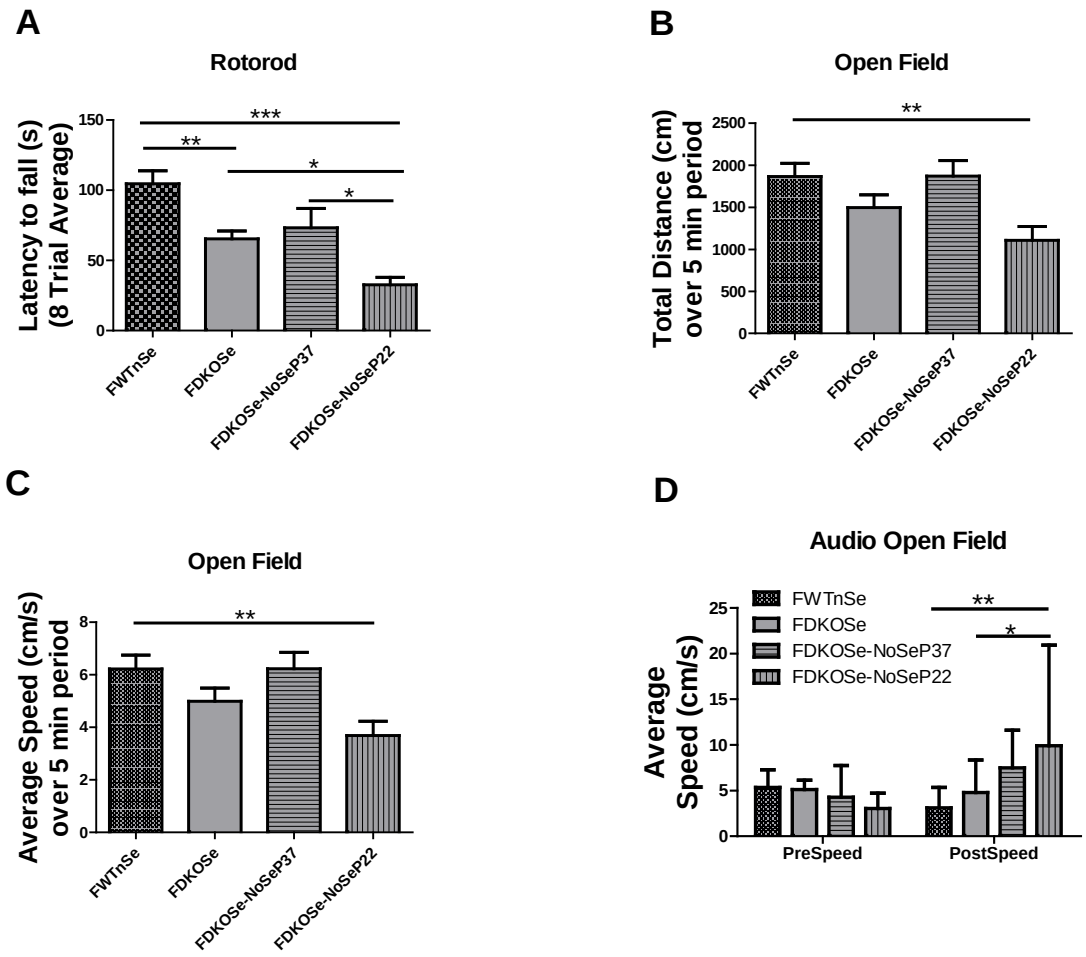



Figure 3.6: Effect of $SeH_2O$ Withdrawal Age on Rotorod, Open Field and Audio Open Field in FDKOSe Mice. Mean latency to fall (A), distance traveled (B) and average speed (C) in open field. Average speed 2½ minutes prior (PreSpeed) and 10 seconds after (PostSpeed) 85 dB white noise played (D) of female WTnSe, DKOSe, DKOSe-NoSeP37 and DKOSe-NoSeP22 mice at 10 weeks of age (One-way ANOVA: Rotorod $p<0.0001$, FWTnSe and FDKOSe n=14, P37 n=7 and P22 n=11; One-way ANOVA: Open Field Distance $p=0.0084$, Ave. Speed $p=0.0084$, FWTnSe and FDKOSe n=12, P37 n=7, P22 n=10; Audio Open Field NS n=11 all groups except FDKOSe-NoSeP37 n=4; Tukey's Multiple Comparison Test *$p<0.05$, **$p<0.005$, ***$p<0.0005$).

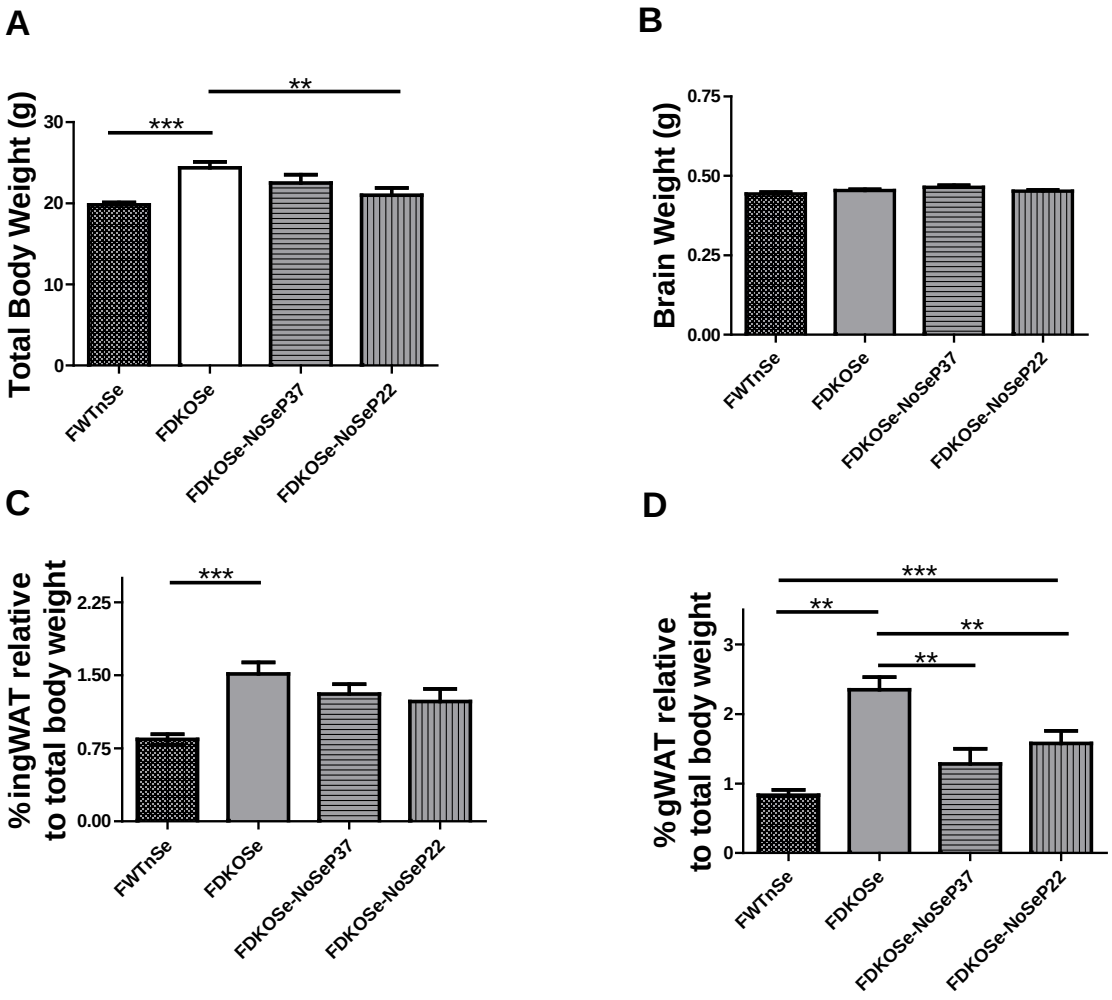



Figure 3.7: Effect of $SeH_2O$ Withdrawal Age on Body Composition of FDKOSe Mice. Body (A), brain (B) and percentage of ingWAT (C) and gWAT (D) of female WTnSe, DKOSe, DKOSe-NoSeP37 and DKOSe-NoSeP22 at 10 weeks of age (One-way ANOVA Total Body p=0.0002, Brain NS, ingWAT% p=0.0005, gWAT% p<0.0001; **p<0.005, ***p<0.0005; n=13 all groups except P37 n=4).

*Assessment of neuroinflammation and neurodegeneration in DKOSe mice*

As the FDKOSe-NoSeP22 neuromotor performance was decreased similar to what has been observed in the MDKOSe in the past, we assessed neurodegeneration in same areas of the brain found to be affected in the MDKOSe, the inferior colliculus (IC) and decussation of the superior cerebellar peduncle (XSCP), along with brain Gpx activity. FDKOSe-NoSeP22 mice exhibited significantly higher levels of GFAP average optical density in the IC and XSCP compared to FWTnSe, FDKOSe and FDKOSe-NoSeP37 (Figure 3.8), indicating substantial

astrocyte reactivity. Silver staining, a stain used to detect degenerating neuronal somata, axons and terminals which become argyrophilic as they deteriorate, showed significant neurodegeneration in the axons of the motor tracts in the XSCP of FDKOSe-NoSeP22 mice (Figure 3.9). When we examined Gpx activity levels there were no significant differences between FDKOSe and FDKOSe-NoSeP22 mice. Both had significantly decreased levels in the whole brain compared to FWTnSe mice. In the brain stem there was a trend of decreased levels with FDKOSe-NoSeP22 having the lowest levels (Figure 3.10).

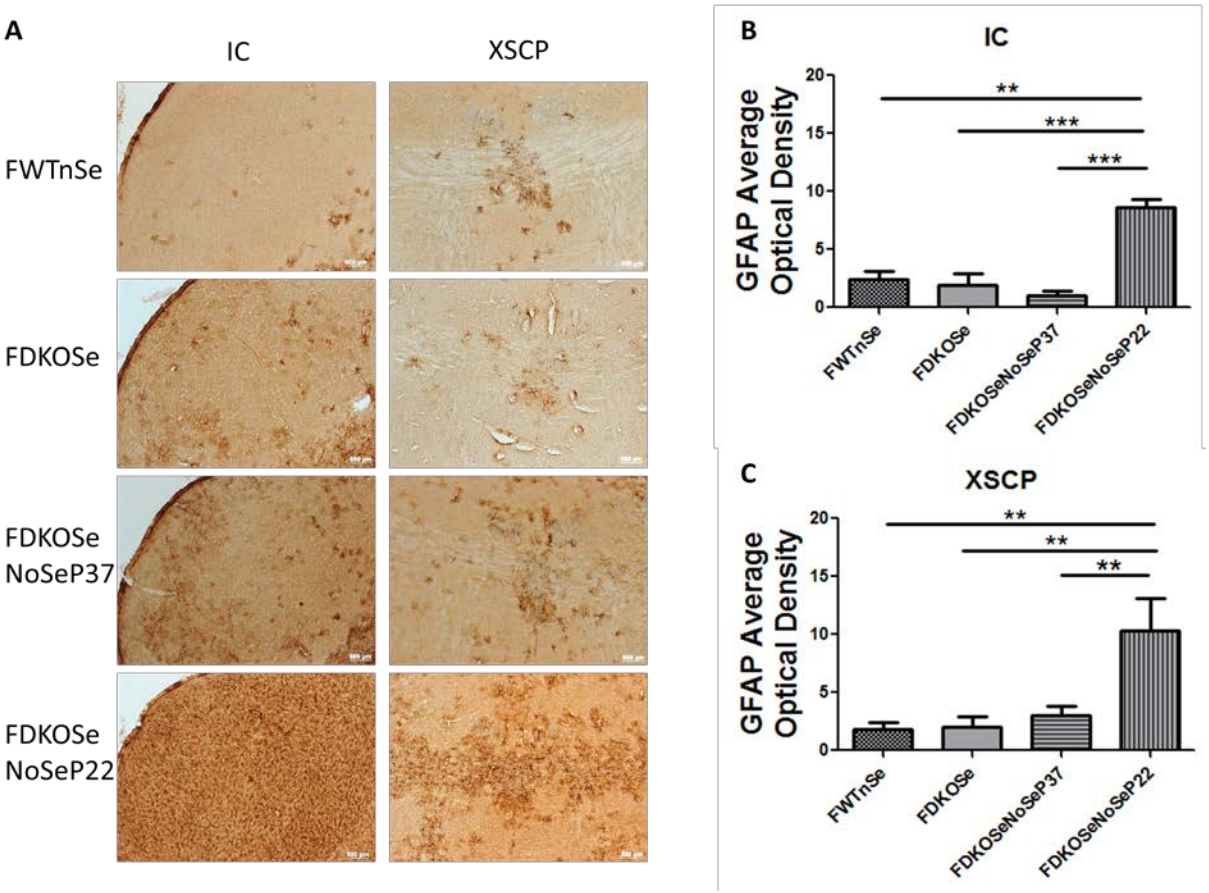



Figure 3.8: Effect of $SeH_2O$ Withdrawal Age on Glial Fibrillary Acidic Protein (GFAP) Immunoreactivity in FDKOSe Mice. A. Representative images of IC and XSCP of FWTnSe, FDKOSe, FDKOSe-NoSeP37 and FDKOSe-NoSeP22 GFAP mice at 10 weeks of age with quantification of average optical density, IC (B) and XSCP (C). (One-way ANOVA IC p=0.0003, XSCP p=0.0151; Tukey's *p<0.5, **p<0.005, ***p<0.0005; n=3 all groups).

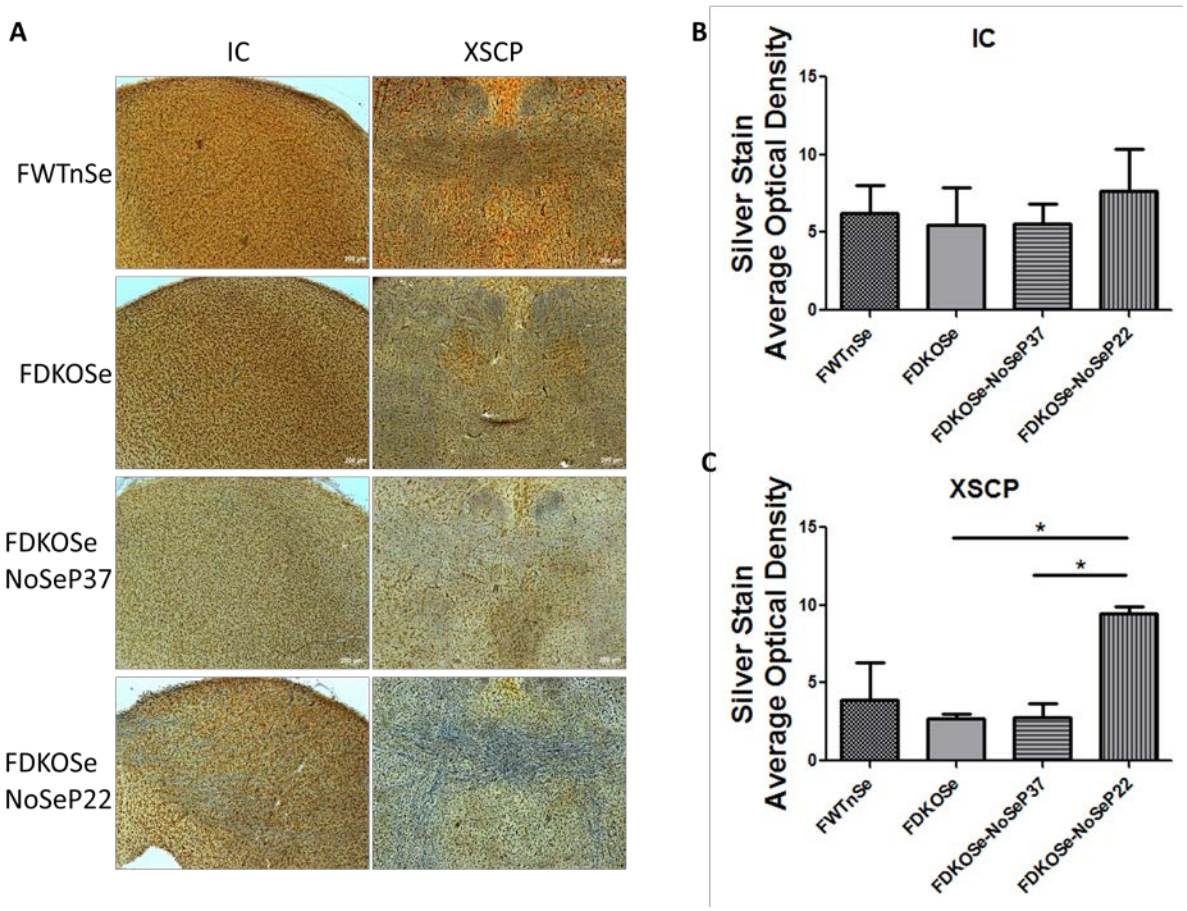



Figure 3.9: Effect of $SeH_2O$ Withdrawal Age on Silver Staining in FDKOSe Mice. A. Representative images of silver staining in IC and XSCP of FWTnSe, FDKOSe, FDKOSe-NoSeP37 and FDKOSe-NoSeP22 mice at 10 weeks of age with quantification of average optical density, IC (B) and XSCP (C) (One-way ANOVA IC NS, XSCP p=0.0187; Tukey's *p<0.5; n=3 all groups).

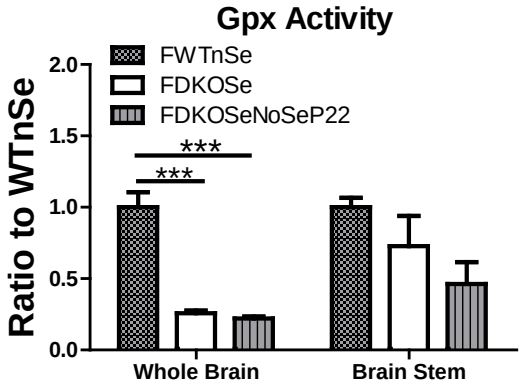



Figure 3.10: Effects of $SeH_2O$ Withdrawal Age on Glutathione Peroxidase (Gpx) Activity Levels in FDKOSe Mice. Gpx Activity in the whole brain and brain stem of FWTnSe, FDKOSe and FDKOSe-NoSeP22 mice at 10 weeks of age. (One-way ANOVA WB $p<0.0001$; BS NS $p=0.0820$; Tukey's Post Hoc Test ****$p<0.0005$, n=3 to 4 in triplicates)

*Impact of the DKOSe genotype on thyroid metabolism*

The FDKOSe neurological phenotype appears to be dependent on $SeH_2O$ supplementation through a specific developmental time point, P37. Selenoproteins play a role in bodily processes such as thyroid hormone (TH) metabolism. TH affects the metabolic activities of most tissues and is important in growth and development. Selenoproteins are also important in oxidative stress defense, which has implications in the development of disease states such as neurodegeneration. We investigated two selenoproteins that might give insight into which of these processes are influenced, brain stem type 3 deiodinase (Dio3) and serum glutathione peroxidase 3 (Gpx3).

Dio3, a critical selenoenzyme in thyroid hormone metabolism, was present in the brain stems of 8 week old MDKOSe mice at almost double the level found in FDKOSe, and was also elevated compared to WT male and female mice (Figure 3.11 A). In FDKOSe mice, Gpx3 was elevated approximately 25% more in the serum compared to MDKOSe mice, and it was

significantly greater compared to wild type females (Figure 3.11 B). These data indicate that there may be a possible TH effect in these mice but as these findings are preliminary further investigations are needed to determine this.

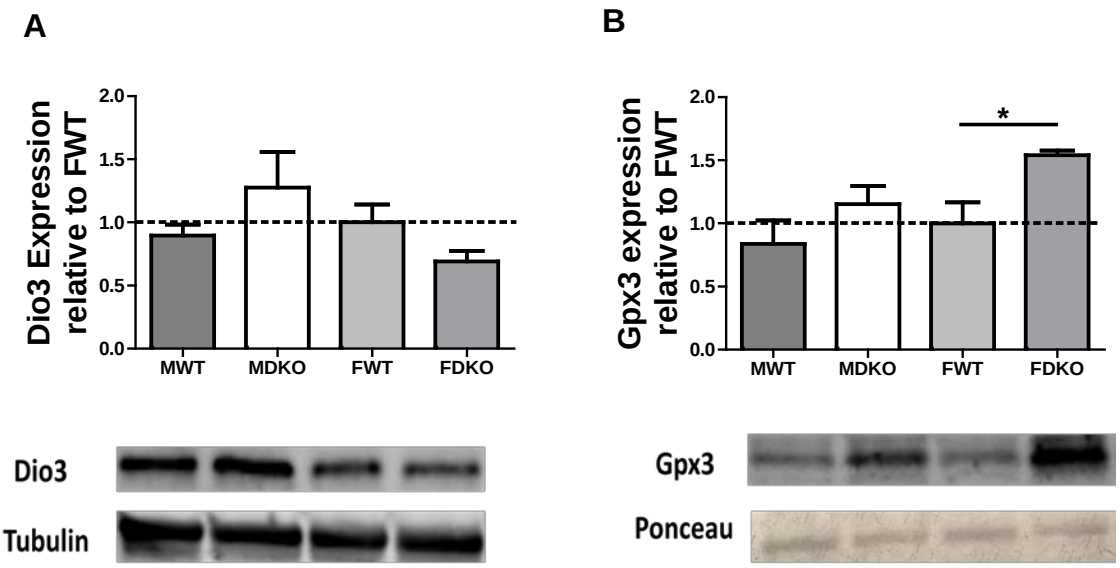



Figure 3.11: Serum Gpx3 and Brain Stem Dio3 Levels in Male and Female DKOSe mice. Mean normalized protein levels relative to FWT in brain stem (A) and serum (B) of male and female WT and DKOSe mice at 10 weeks of age. (Two-way ANOVA: Dio3 NS; Gpx3, Genotype $F_{(1,12)}$ =8.64 p=0.0124, Sex $F_{(1,12)}$ =3.59 p=0.0826, Bonferroni post-test *p<0.05, n=4 all groups).

**Discussion**

In the previous study characterizing DKOSe mice, the severe phenotype of MDKOSe was not observed in FDKOSe (2). Herein, we confirm that FDKOSe mice exhibit a milder neurological phenotype. FDKOSe mice have decreased Rotorod performance starting at seven weeks of age, a week later than MDKOSe mice. A significant reduction in performance compared to FWTnSe was observed when they reached nine weeks of age indicating that, though milder, this condition was progressive.

$SeH_2O$ supplementation until at least P37 appears to be critical in preventing the more severe consequences observed in MDKOSe from occurring in FDKOSe mice. With $SeH_2O$ removal at weaning (P22), FDKOSe mice developed a phenotype similar to that seen in MDKOSe mice. They had significant decreases in motor coordination and locomotion, along with increased susceptibility to seizure development. Neuroinflammation and neurodegeneration were evident in the same areas of the brain, IC and XSCP, as previously observed in MDKOSe mice, indicating the possibility that the same developmental factors and/or mechanistic pathways are disrupted in both males and females.

MDKOSe mice develop increased susceptibility to audiogenic seizures (AGS) with GABAergic system defects including decreased $GAD_{67}$ and $PV^+$IN density in the IC (2). Other mouse models of AGS have shown that GABAergic system defects during a critical postnatal development period of P12-22 is a factor in rodent susceptibility to audiogenic seizures (AGS). This may be due to mouse brain development at this time. It is estimated that onset of myelination in IC occurs at about P12 and auditory radiations and PV peaks at P16. Studies have shown that GABA deficient mutant animals have lower seizure threshold and/or spontaneous seizures. In propylthiouracil (PTU) audiogenic seizure mice, administration of PTU (an anti-thyroid drug) at 0-19 days postnatal causes hypothyroidism during that time which is believed to cause GABA circuit defects in the mice, rendering them susceptible to audiogenic seizures (AGS) starting at around 7 weeks. Administration of a GABA agonist/positive modulator was able to restore GABA inhibition in adolescence. To determine if FDKOSe mice develop seizures similarly to MDKOSe, studies on $PV^+$IN and GABAergic system defects in these brain areas need to be conducted.

Even with $SeH_2O$ supplementation, FDKOSe mice showed increased percentage of ingWAT and gWAT deposits compared to their FWTnSe counterparts, but no significant

differences in fasting insulin levels. If insulin secretion was affected, we expected there to be lower levels of insulin present in the groups that are defective. But, if the glucose uptake mechanism is affected, then insulin levels may appear elevated or comparable between the groups. As the fasting insulin levels were comparative between the groups a follow-up of fasting glucose and/or glucose tolerance test may need to be conducted to confirm the later affect.

In mammals, white adipose tissue is used for energy storage in the form of lipids and is considered one of the largest endocrine organs involved in both immunity and metabolic mechanisms (108; 109). An increase in subcutaneous fat normally occurs prior to abdominal (visceral) deposits (110) and has been shown to be an independent predictor of type 2 diabetes without the presence of insulin resistance (IR) (111). Visceral obesity is an important determinate of the development of pathogeneses of chronic inflammation and insulin resistance (IR) leading to metabolic syndrome and increased risk of type 2 diabetes mellitus and cardiovascular disease development (108; 109; 112). The increased fat deposits in FDKOSe could be evidence of the beginning of a metabolic deficit that appears mild at this age, under the current diet but further testing would be needed to determine this.

In addition, the elevated serum Gpx3 levels in FDKOSe mice may also indicate the beginning of a metabolic deficit (Figure 3.11). Gpx3 is an extracellular glutathione peroxidase that accounts for about 30% of Se in the plasma (113; 114). It has been found to be synthesized and secreted into the follicular lumen of the thyroid. In the follicular lumen it is thought to modulate hydrogen peroxide ($H_2O_2$) supply, the rate limiting co-factor in TH biosynthesis, and catalyze thyroglobulin polymerization into a highly cross-linked, storage form (74; 115; 116). It is possible that Gpx3 may be having an effect on TH by modulating its synthesis and storage but further investigation would be needed to evaluate this possibility.

Alternatively, Gpx3 has also been implicated as a status marker in development of metabolic syndrome as increased serum levels of Gpx3 have been found in patients with this disorder (117). In FDKOSe mice both ingWAT and gWAT deposit percentages are significantly increased. Since increased subcutaneous fat deposits even without evidence of IR has been indicated to precede metabolic syndrome development, this seems to be the more likely explanation for the increased Gpx3 levels in these mice. The mild phenotype may be due to the relatively young age of the mice tested and, as metabolic syndrome severity tends to increase

with age, a longer observation may be beneficial in confirming whether further metabolic disruption would develop.

**Conclusion**

Based on the data presented above, it is apparent that FDKOSe mice have a neurological deficit which becomes severe leading to seizure development if $SeH_2O$ is removed prior to age P37. When $SeH_2O$ supplementation is removed at weaning, 83% of FDKOSe developed a severe neurological phenotype by 10 weeks of age. FDKOSe mice had significantly increased percentage of ingWAT and gWAT fat deposits as well as serum Gpx3 levels which may indicate a metabolic component but it appears to be mild at this age as fasting insulin levels were unaffected compared to FWTnSe mice. Together, these results indicate that there is a window of development in which Se is important for FDKOSe mice to prevent severe neurological deficits.

# Chapter 4: Competition between the Brain and Testes for Se in MDKOSe mice

## Introduction

Se distribution in the body varies by tissue, with priority given to the brain and endocrine tissues (107), and this is also sexually dimorphic. Females have been shown to have higher retention in all tissues except the brain, where it is similar in both sexes, and the reproductive organs, where retention is higher in the testes compared to ovaries (76; 107). The similar Se brain levels appears to change in the DKOSe mice, as the Se concentration was found to be about 27% higher in the females than males (Figure 4.1) which could be due to a change in retention, availability or turnover.

Testosterone is an androgen produced by the adrenal cortex and gonads that increases in concentration in males during puberty is testosterone (118). It primarily affects the maturation and function of secondary sex organs, determination of male sexual characteristics, and is involved in tissue and muscle growth. Males have three surges of testosterone production during development. In rodents, these occur before birth, right after birth and at puberty (119). MDKOSe mice have decreased survival, neurological dysfunction and onset of susceptibility to AGS, which occur around puberty at 8 weeks of age the same time as the reported testosterone surge to adult levels. Thus, we hypothesized that testosterone could possibly be an agonist in the production of the MDKOSe neurodegenerative state via its ability to increase cell metabolism and thereby oxidative stress.

In males, greater than 95% of testosterone is made in the testes. Therefore, in order to determine if testosterone could be a factor in the MDKOSe neurological deficit development, we first castrated the male DKOSe, Sepp1KO, and WT mice and tested them for neurological impairment. Then, we re-administrated testosterone to a group of castrated and sham operated mice to determine if testosterone could reverse the effects of castration and/or alter the MDKOSe phenotype.

**Hypothesis:** Testosterone supplementation may exacerbate or potentially ameliorate the MDKO neurodegenerative phenotype.

**Aim:** Determine the neurological effects of testosterone in the MDKOSe phenotype.

**Significance:** Identifying the underlying factors and compensatory processes that are distinct in males versus females during childhood and adolescent development may provide new insights into potential therapeutic targets in cases of fetal and childhood Se deficiency.

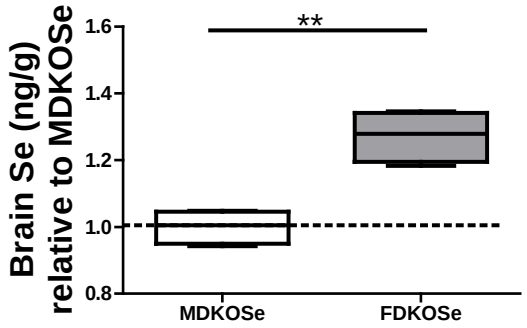



Figure 4.1: Se Brain Concentrations in DKOSe Mice. Brain Se levels in male and female DKOSe relative to MDKOSe (n=4, Student T-test **p=0.0011)

**Materials and methods**

*Animals:* Mice were generated, maintained, and samples collected as previously described in Chapter 2 with the exception of WT mice used in the MK-801 study. These mice were group housed until 8–10 weeks of age and then single housed 5 days before the onset of experiments. Behavioral testing was conducted on adult mice between the ages of 9 and 15 weeks during the light cycle.

*Diet:* A select group of animals were administered a low Se diet of 0.01 ppm selenium beginning at either weaning (WT MK-801 experiments) or at 10 weeks of age (Sepp1KO experiments).

*Castration and Sham Surgery:* Four to five week old MWTnSe and MDKOSe mice were anesthetized and surgical area prepared. An abdominal incision was made near the groin area, the

tunica was pierced and the testes along with epididymis were exposed and removed. The incision was then sutured closed and animals received postoperative analgesic administration. Sham animals underwent the same procedure but testicles and epididymis were not removed.

*MK-801 drug treatment:* At 6 weeks of age, select WT mice received intraperitoneal injections of PBS or MK-801 (Sigma-Aldrich; 0.2 mg/kg in PBS) for 5 consecutive days.

*Histology and immunohistochemistry:* All were conducted as described in Chapter 3 except for in immunofluorescence the appropriate AlexaFluor-labeled fluorescent secondary antibodies were used for visualization instead of DAB. Perineuronal nets (PNNs) were labeled by incubation in a solution containing biotinylated Wisteria floribunda lectin (1:1000; Sigma, L1516), washed, and then incubated with fluorescent avidin (1:500; Vector Labs) for visualization.

*Silver staining:* Silver staining was performed as described in Chapter 3.

*Protein extraction and immunoblotting:* All were conducted as described in Chapter 3 except relative protein levels were determined by dividing the optical density of the band representing the protein of interest by that of α-tubulin (loading control). The mean normalized optical density for each protein of interest in sham DKOSe mice was recalibrated to equal 100.

*Antibodies:* The primary antibodies used for immunohistochemistry were as follows: mouse anti-GAD67 (1:20,000; EMD Millipore, MAB5406), rabbit anti-PGC-1 (1:200; Santa Cruz Biotechnology SC-13067), rabbit anti-GFAP (1:2000; DAKO Z0334), goat anti-ApoER2 (1:50; Santa Cruz Biotechnology SC-10113), rabbit anti-selenophosphate synthetase 2 (1:1000; Rockland 200-401-999), rabbit anti-parvalbumin (1:5000; Swant PV 25), and rabbit anti-c-Fos (1:5000; EMD Millipore PC38). The primary antibodies used for Western blotting were as follows: mouse anti-α-tubulin (1:5000; Novus Biologicals NB100-690), rabbit anti-PGC-1 (1:1000; EMD Millipore AB3242), goat anti-glutathione peroxidase 1 (1:500; R&D Systems AF3798), rabbit anti-GPx4 (1:1000; Cayman Chemical 10005258), and rabbit anti-SelM (1:500; Sigma-Aldrich HPA019601).

*Gpx activity assay:* Gpx activity was measured as described in Chapter 3

*Neuromotor and Behavioral tests:* All analyses were conducted as described in Chapter 2 except the Open Field test for the testosterone re-administration group. The 2½ minute acclimation period was used in lieu of conducting a separate test.

For the WT MK-801 study mice underwent the following additional test:

- Barnes Maze test for spatial learning and memory. Assay was conducted as described previously (120). In brief, the maze consists of 40 equally spaced wholes on a white circular board, with a removable escape tunnel attached to one whole. Mice were trained to find the escape tunnel with two 3 minutes trials for 10 days. If a mouse failed to find the escape tunnel within the 3 minute period, it was placed in the escape tunnel by the researcher and allowed to stay there for 15 seconds. The latency to locate the escape tunnel and the number of incorrect holes checked (errors) before locating the escape tunnel were recorded.

*Se analysis:* Se was measured using a modification of the fluorometric assay of Koh and Benson (1983) and Sheehan and Gao (1990). Briefly, tissue was predigested in nitric acid at 150°C followed by digestion with perchloric nitric acid (1:4) solution at 190°C over a 1½ hour time period. Solution cooled to 150°C, as it cooled 0.5 ml of concentrated HCl was added, samples were then maintained between 125°C and 150°C for 30 minutes. Next, 2 ml of 0.1 M EDTA and 0.5 ml of 2, 3-diaminonaphthalene were added, followed by 3 ml of cyclohexane and samples allowed to incubate at 60°C for 30 minutes. Fluorescence was then measured by a PerkinElmer LS 55 fluorometer and concentrations determined via comparison with a standard curve.

*Testosterone measurement:* Blood was collected from adult mice via cardiac puncture during the early evening period (4:00–6:00 P.M.). Serum testosterone levels were then determined by means of a testosterone ELISA kit (Crystal Chem) pursuant to the manufactures instructions.

*Testosterone and Placebo Administration:* Testosterone 60 day releasing pellets at 25 µg/day and placebo pellets (Innovative Research of America, Testosterone and matching Placebo 1.5 mg 60 day releasing pellets catalog # SA-151 and SC-111) were administered via subcutaneous insertion at between 5 to 6 weeks of age.

*Stereology:* Analysis of cell density was performed on a Zeiss microscope (Axioskop2) equipped with Stereo Investigator software (MBF Bioscience). An optical dissector (counting box) was used to count cells. The retrosplenial granular cortex and IC were outlined using a 5 x objective at specified levels relative to bregma (retrosplenial granular cortex -1.46 mm, IC -5.02 mm). Optical fractionator analysis was conducted at high magnification (20 x objective) using a 300 x 300 µm counting frame to quantify the number of positively labeled cells. At each

counting site, the mounted section thickness was determined. Mean section thickness was calculated by averaging all counting sites, and this value was used to determine the number of positively labeled cells per unit volume. For double-label experiments, parvalbumin (PV)-positive cells (red) were counted first then the filter was switched, and WFA-positive cells (green) were counted by revisiting the same sites previously sampled. Finally, counting sites were revisited again and cells with overlapping markers (PV, WFA) were counted as PNN-labeled PV interneurons.

*Quantification of immunohistochemistry and silver staining:* Analysis were conducted as previously described in Chapter 3.

*Statistical analysis:* All were conducted as described in Chapter 2.

**Results: Castration of Male DKOSe, Sepp1KO and WT Mice**

*MDKOSe mice*

We found that castration prevented severe neurological dysfunction in MDKOSe mice. Castrated MDKOSe mice had 100% survival at 10 weeks (Figure 4.2 A) with significant increases in motor coordination and locomotion (Figure 4.2 B and C). They failed to develop audiogenic seizures (Figure 4.2 D). When we examined the areas where neurodegeneration is normally observed in these mice (i.e. the inferior colliculus (IC) and decussation of superior cerebellar peduncle (XSCP)), neurodegeneration was not observed (Figure 4.3). Brain Se levels were significantly increased in adult castrated mice (Figure 4.4 A), as well as, Gpx activity in both the whole brain and brain stem (Figure 4.4 B). Castration seems to have promoted the maturation of parvalbumin expressing interneurons ($PV^+IN$) as detected by a higher percentage of $PV^+IN$ enclosed by perineuronal nets (PNNs) (Figure 4.5), which are specialized extracellular matrix that enwrap $PV^+IN$ as they mature, thereby promoting interneuron maturation and synaptic network stability.

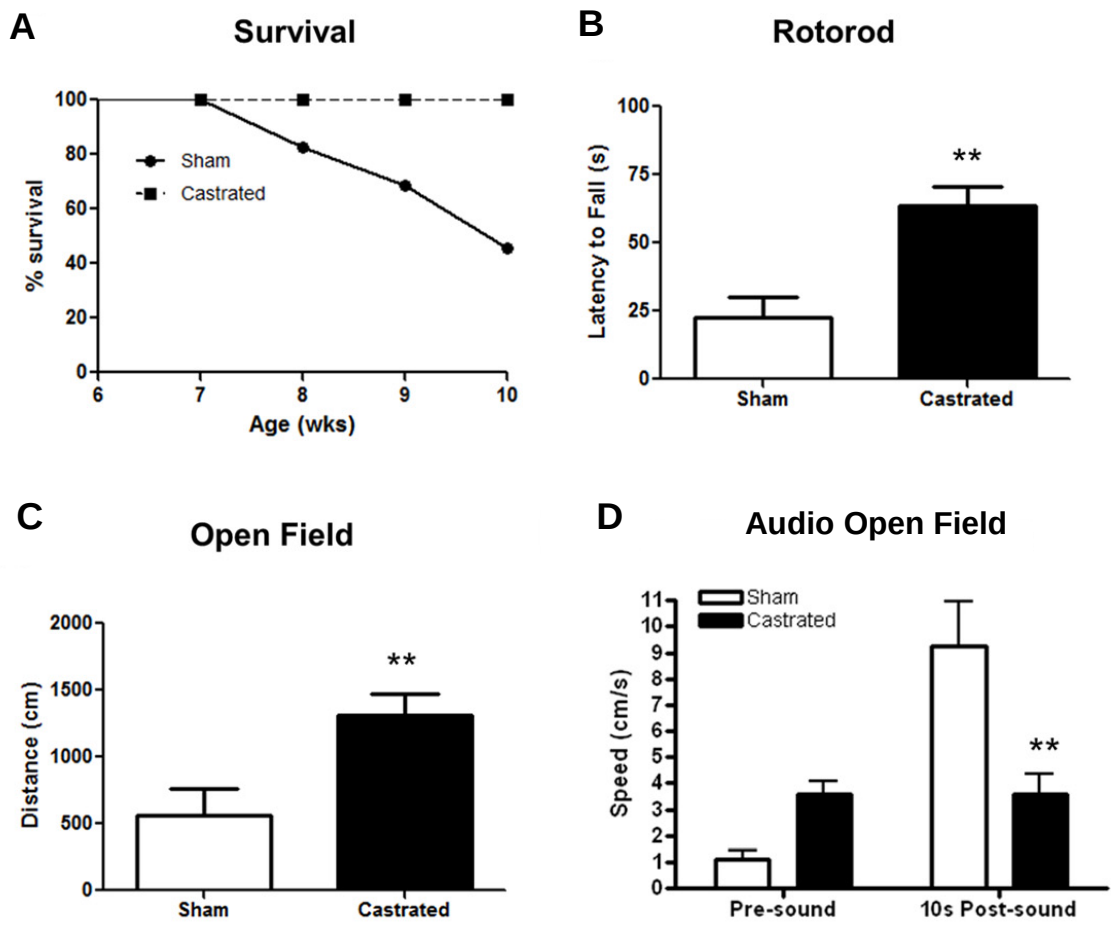



Figure 4.2: Percent Survival and Rotorod, Open Field and Audio Open Field Behavior in Castrated MDKOSe Mice. A. Survival curve of sham (n=31) and castrated (n=20) DKOSe mice. B. Mean latency to fall off rotorod (Student t-test: $t_{(22)}$=3.422 **p<0.01; sham n=7, castrated n=17). C. Mean distance traveled during the open field test (Student t-test: $t_{(20)}$=2.886 **p<0.01; sham n=9, castrated n=13). D. Mean speed before 85 dB white noise and in the 10 second period after start of 85 dB white noise (Two-way ANOVA: Interaction $F_{(1,19)}$ =22.69 p<0.001; Sound $F_{(1,19)}$=725.51 **p<0.001; Castration $F_{(1,19)}$=7.232 p<0.05; sham n=5, castrated n=16).

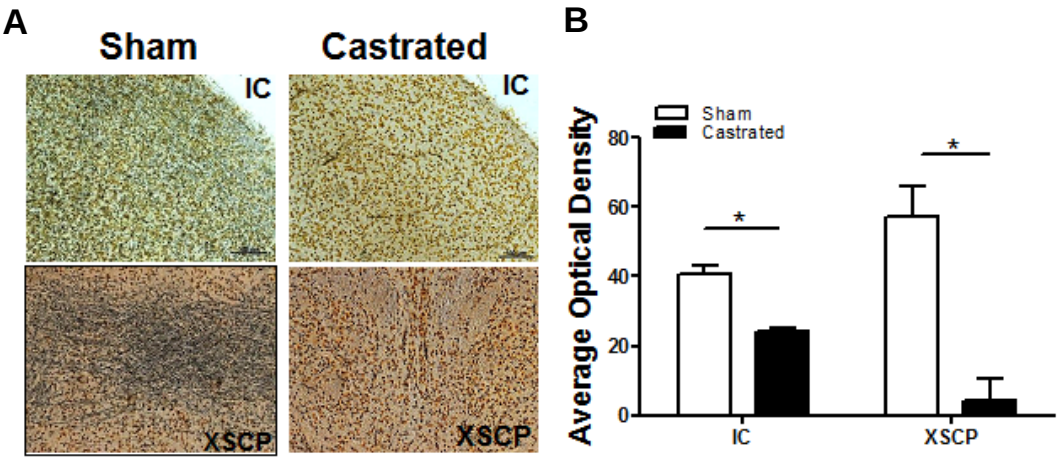



Figure 4.3: Silver Staining Density in Castrated MDKOSe Mice. A. Representative images (20x) of silver stained sections from IC and XSCP of sham (left) and castrated (right) MDKOSe mice; B. Quantification of average optical density of IC and XSCP of sham (n=3) and castrated (n=3) (Student t-test: IC $t_{(4)}$=3.422 *p=0.0052; XSCP $t_{(4)}$=4.906 *p=0.0080).

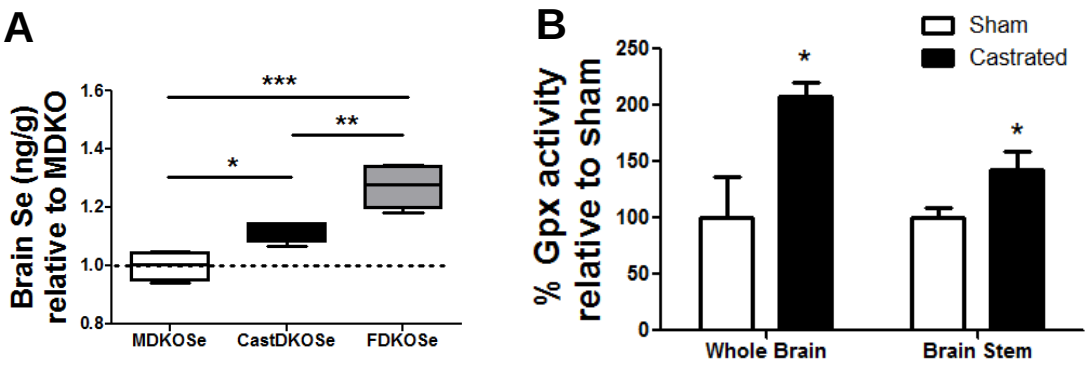



Figure 4.4: Brain Selenium and Gpx Activity Levels in Castrated MDKOSe Mice. A. Mean (±SEM) whole brain Se levels in male (*n*=4), castrated (*n*=5), and female (*n*=4) *DKOSe* mice (One-way ANOVA p=0.0001, Tukey's post hoc *,** and *** p=<0.05); B. Mean (±SEM) normalized GPx activity in whole brain (*n*=4 or 5 per group) and brainstem (*n*=8 per group) of sham and castrated DKOSe mice, samples relative to sham samples. (Student t-test: whole brain $t_{(7)}$ =3.037, *p<0.05; brainstem $t_{(14)}$ =2.235, *p<0.05)

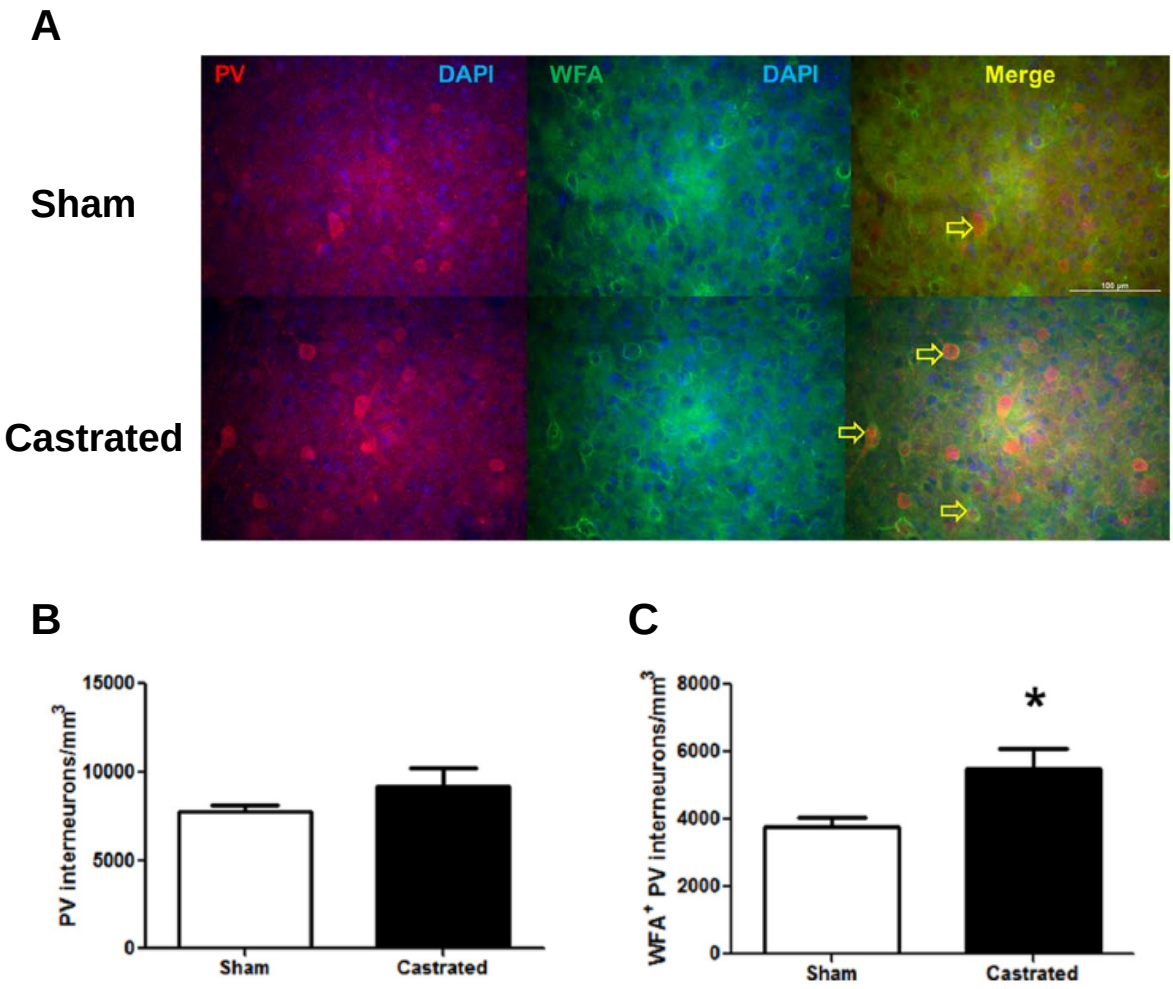



Figure 4.5: Parvalbumin Expressing Interneurons in Castrated MDKOSe. A. Representative immunofluorescent images of PV (red) and PNNs (WFA-labeled, green) from the IC of sham and castrated *DKOSe* mice. Yellow arrows indicate WFA-labeled PV interneurons in merged images. B. Mean (±SEM) density of PV interneurons per mm3. C. Mean (±SEM) density of PNNs (WFA-labeled) PV interneurons per mm3 (Student t-test: $t_{(7)}$=2.446, *$p$<0.05; sham: $n$=4, castrated: $n$=5; Scale bar, 100 μm).

*MSepp1KO mice*

When administered a Se-deficient diet, Sepp1KO mice develop neurological dysfunction (93). Castration of Sepp1KO mice on a Se-deficient diet appeared to reverse this effect. Castrated Sepp1KO mice exhibited significantly better Rotorod performance than sham mice after five weeks of a Se-deficient diet (Figure 4.6 A) and none exhibited wild running nor seizure

response when tested in the AOF (Figure 4.6 B). The IC and XSCP had significantly less silver staining, indicating neurodegeneration was diminished in these brain regions (Figure 4.7 A and B). Gpx activity was significantly higher in the brain stem of castrated Sepp1KO mice compared to the sham operated mice with activity trending higher in the brain as a whole (Figure 4.7 C). Though castration appeared to be protective under Se-deficient conditions in these mice, this effect was not long-lasting as onset of neurological dysfunction was only delayed by three weeks.

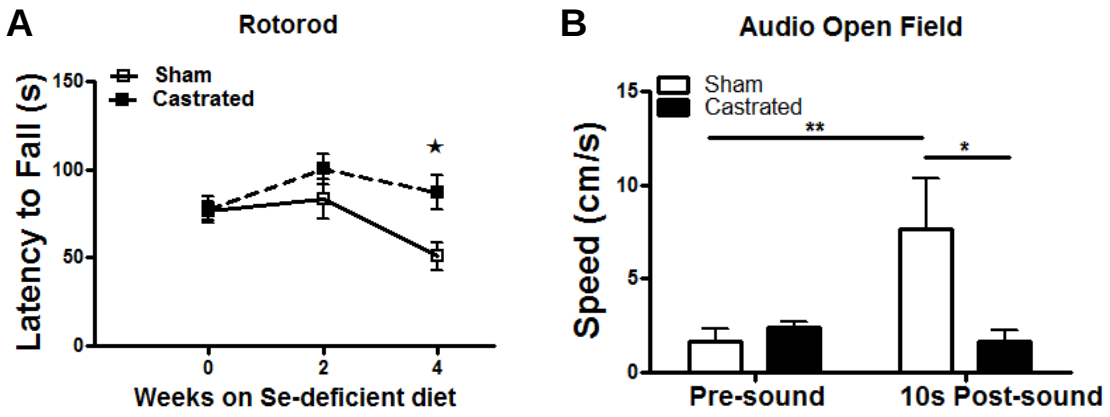



Figure 4.6: Weekly Rotorod and Audio Open Field Behavior in Castrated MSepp1KO on Se-deficient Diet. A. Castration delays neurological impairment and neurodegeneration in Sepp1KO mice challenged with a Se-deficient diet. A. Mean (±SEM) latency to fall off rotorod from 0 to 4 weeks on a low Se diet (Two-way ANOVA: time $F_{(2,36)}$=6.661, p<0.01; interaction $F_{(2,36)}$=3.788, p<0.05; Week 4 Student t-test: $t_{(18)}$=2.954, *p=0.0106, *n*=10 per group) and (B) Mean (±SEM) speed before 85 dB white noise and in the 10 seconds period immediately after start of 85 dB white noise after 5 weeks on a low Se diet (*n*=10 per group) of castrated and sham Sepp1KO mice. (Two-way ANOVA: interaction $F_{(1,18)}$=5.836, **p< 0.05; Post-sound $t_{(18)}$=2.990, *p<0.05).

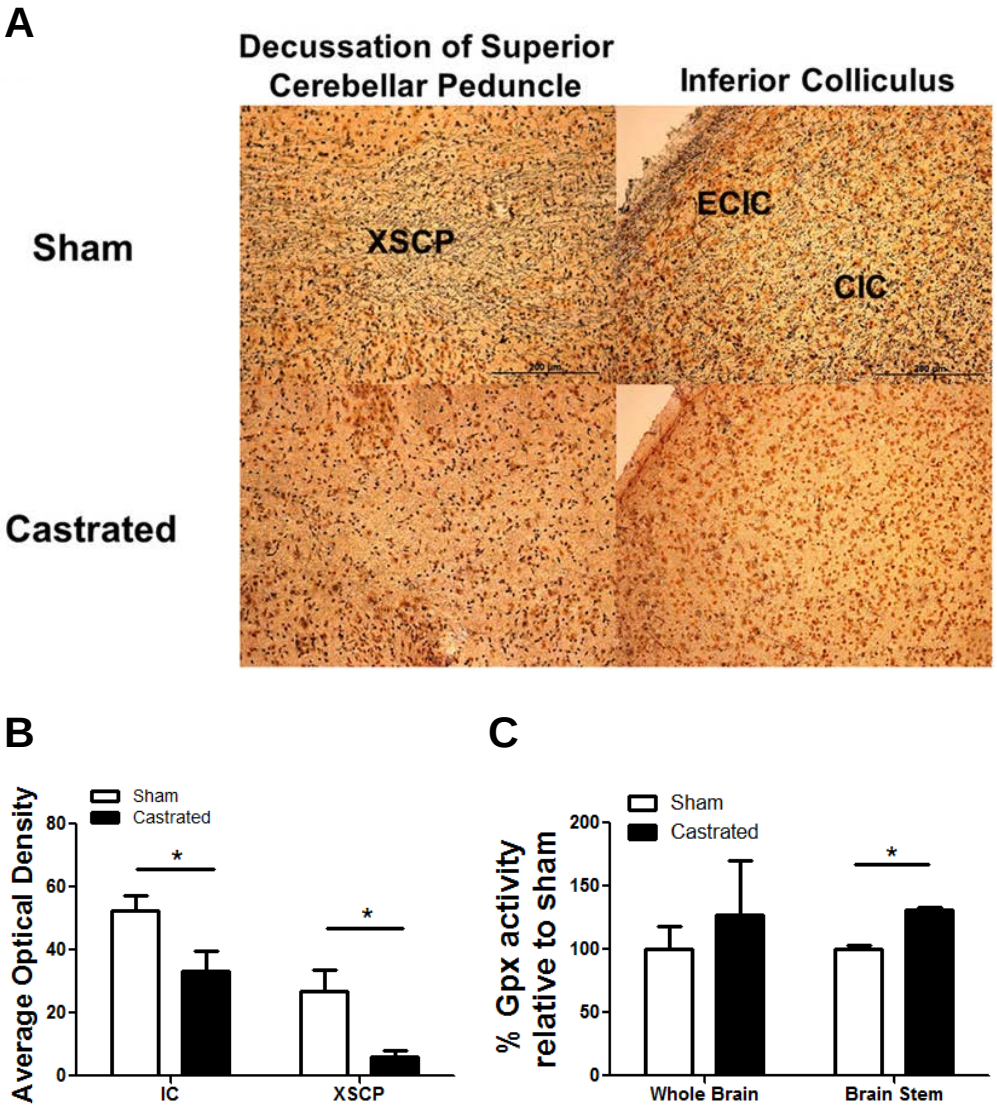



Figure 4.7: Silver Staining and Gpx Activity Levels in Castrated Sepp1KO Mice on Se-deficient Diet. A. Representative images of silver-stained brain sections containing the XSCP (left) and the IC (right); Scale bar, 200 μm. B. Mean (±SEM) optical density of silver staining in sham (*n*=5) and castrated (*n*=4) Sepp1KO mice (Student t-test: IC $t_{(7)}$= 2.415,* p< 0.05; XSCP $t_{(7)}$=2.671, *p<0.05). C. Mean (±SEM) normalized GPx activity in brainstem and whole-brain samples (Student t-test: brain stem $t_{(4)}$=7.340, *p<0.01, n=6 per group). (*CIC=Central nucleus of inferior colliculus; ECIC=external cortex of inferior colliculus*.).

*MWT mice*

In addition to DKOSe and Sepp1KO mice, we castrated wild type mice (WT) and exposed them to dizocilpine maleate (MK-801) at puberty. MK-801 is a non-competitive NMDA receptor antagonist which would elevate oxidative stress, causing neurotoxicity. It has also been reported to impair maturation of GABAergic neurons (121). Castrated WT MK-801 mice had improved spatial learning, as tested in the Barnes maze (Figure 4.8), and trended to better performance on the Rotorod (Figure 4.9 A) compared to sham WT. Their Gpx activity levels were significantly elevated (Figure 4.9 B) and $PV^{+}$IN density was increased in the regions the retrosplenial cortex normally affected by the MK-801 neurotoxin (Figure 4.10).

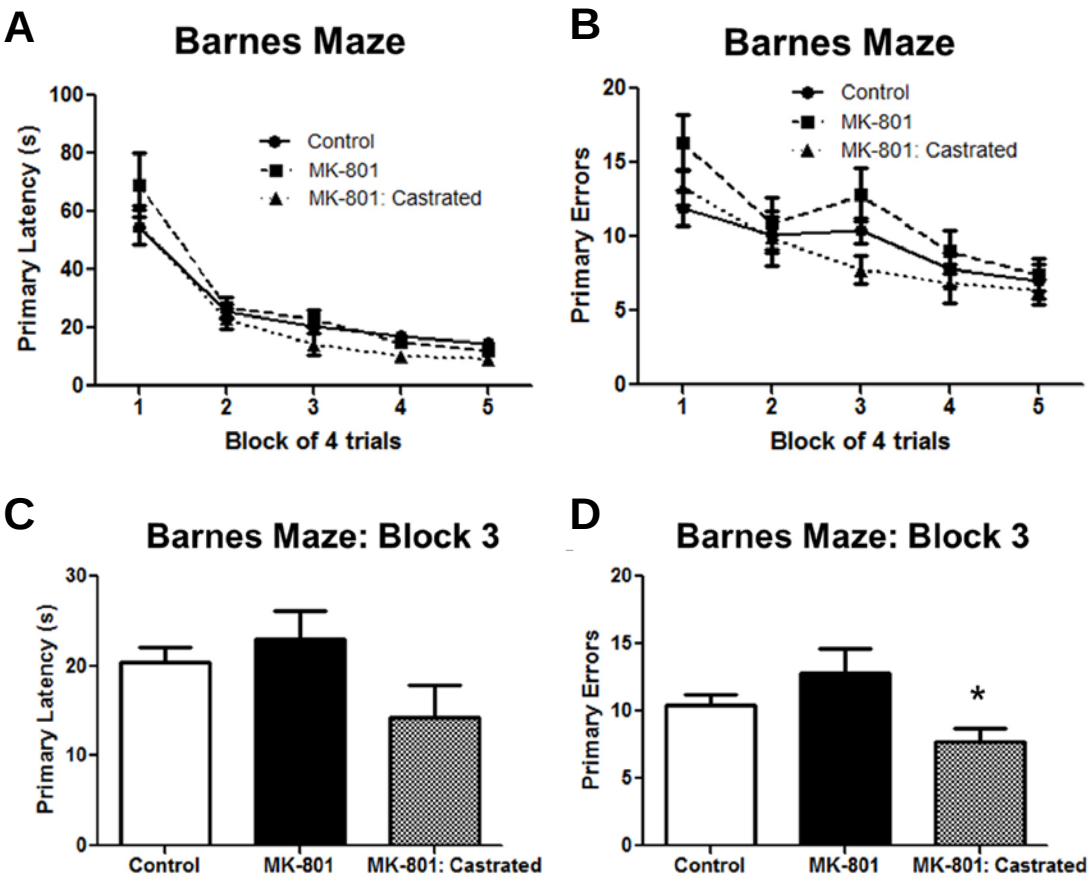



Figure 4.8: Barnes Maze Behavior in Castrated WT Mice Administered MK-801. A. Mean (±SEM) latency to locate the escape tunnel during Barnes maze training (Two-way ANOVA: primary latency $F_{(4,108)}$= 71.87, p< 0.0001). B. Mean (±SEM) number of incorrect holes checked before locating the escape tunnel during training (Two-way ANOVA: primary errors $F_{(4,108)}$=71.87, p<0.0001). C. Mean (±SEM) latency to locate the escape tunnel during trial block 3 (p<0.1123). D. Mean (±SEM) number of incorrect holes checked before locating the escape tunnel during trial block 3 (One-way ANOVA: primary errors $F_{(2,27)}$=3.930, *p<0.05 compared with MK-801 group; *n*=10 per group).

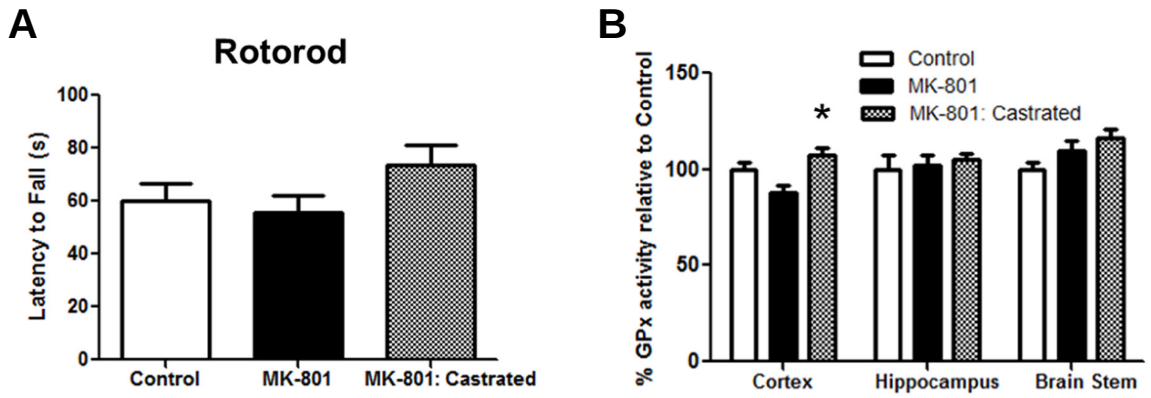



Figure 4.9: Rotorod Behavior and Gpx Activity Levels in WT Mice Administered MK-801. A. Mean (±SEM) latency to fall off Rotorod. B. Mean (±SEM) normalized GPx activity in samples from cortex, hippocampus, and brainstem (One-way ANOVA: Cortex $F_{(2,11)}$=6.440, *p< 0.05; *n*=4 per group).

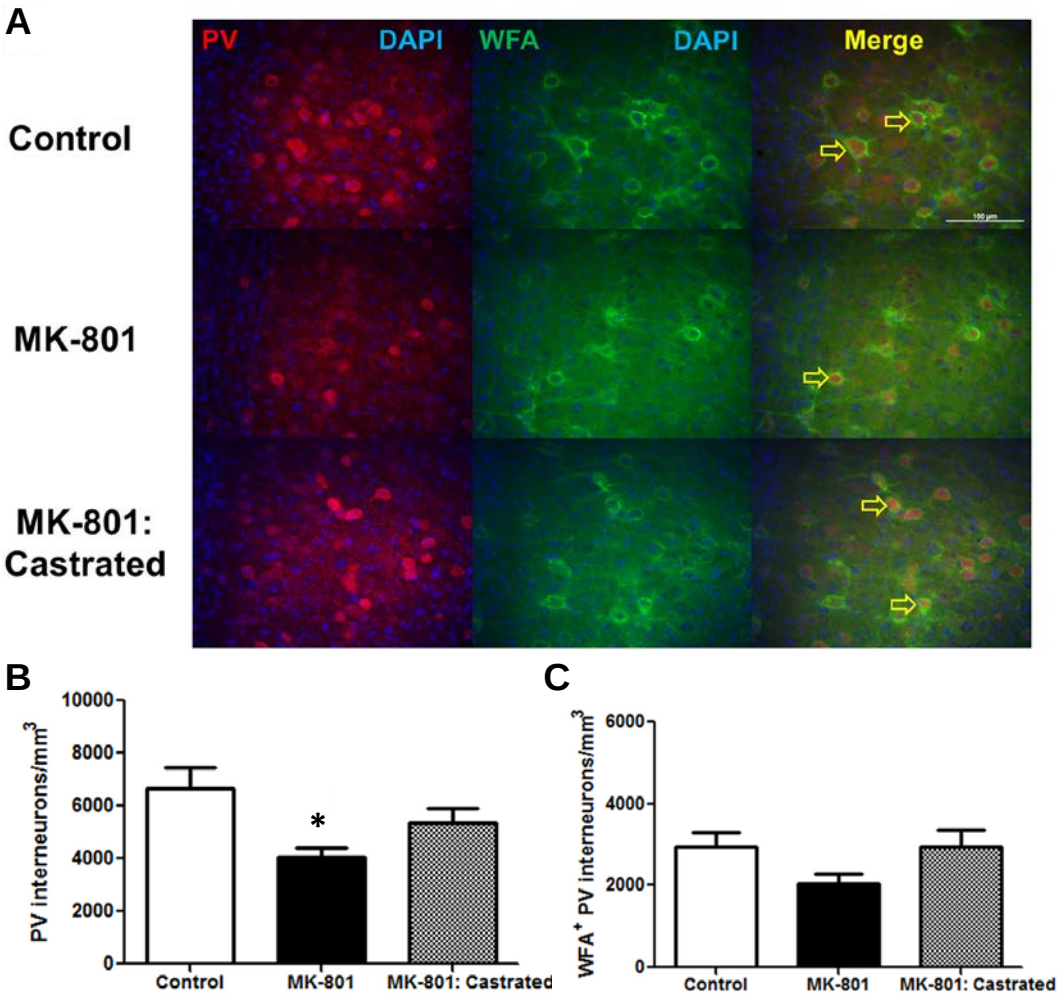



Figure 4.10: Parvalbumin Expressing Interneurons in WT Mice Administered MK-801. A. Representative immunofluorescent images of PV (red) and WFA (green) from the retrosplenial cortex. Yellow arrows indicate WFA-labeled PV interneurons in merged images. B. Mean (±SEM) density of PV interneurons per mm3 (One-way ANOVA: $F_{(2,15)}=4.817$, *$p<0.05$ compared with Control group). C. Mean (±SEM) density of WFA-labeled PV interneurons per mm3. Scale bar, 100 μm.

Thus, castration seems to be neuroprotective. It appears to rescue the MDKO phenotype, delay neurological effects of a Se deficient diet in MSepp1KO mice, and provides partial protection from the neurotoxin, MK-801, in WT mice. The main neurological effect of castration observed was the promotion of $PV^{+}$IN maturation thereby promoting normal excitatory/inhibitory balance. This finding, along with the increased Se concentration and Gpx activity in the brain, indicate that the brain and testes may compete for Se supply in the absence of Scly and Sepp1, which may lead to the male sex specific phenotype.

**Results: Testosterone Re-administration in MDKOSe Mice**

Upon further characterization of the MDKOSe mice, we found that disrupted Se metabolism appears to be preceded by signs of reactive gliosis at 4 weeks of age (data not shown), weeks prior to the apparent GABAergic system deficits and loss of PV expression seen in adult MDKOSe mice at 8 weeks of age. This early onset of disruption in certain brain areas has been previously reported in Sepp1KO mice (122). As stated above, castration seems to rescue the MDKOSe phenotype and delays the onset of motor dysfunction in MSepp1KO mice on a Se deficient diet. When investigating Se levels, we found that castration appears to significantly increase Se concentrations in the brains of MDKOSe mice (Figure 4.4 A) but when comparing Se concentrations in the testes, although MDKOSe had significantly reduced Se levels compared to MWT, Se levels were not significantly different from those found in male Sepp1KO that do not develop a neurological phenotype (Figure 4.11). Therefore, the apparent protection from castration seems to be due to increased Se availability in the brain (102) but the permanent susceptibility to Se deficiency in these mice implicates a developmental process that is possibly being disrupted prior to weaning at 4 weeks of age.

Testosterone has been shown to reverse the decreased cellular metabolism in castrated rats (123). The effect of testosterone may increase/accelerate the degeneration in the MDKOSe mice due to increased oxidative stress associated with increased metabolism. This would be manifested in reversing the protective effects of castration in neuromotor and behavior tests. A detrimental effect of testosterone replacement would have implications in hormone replacement therapies in males with compromised anti-oxidative defense systems, as oxidative status may need to be taken into consideration prior to prescribing testosterone as a treatment. Alternatively,

if testosterone replacement does not exacerbate the neurological phenotype, this would imply that the effects of castration stem from eliminating competition for Se between brain and testes.

**Hypothesis:** Protective effects of castration are due to either or both of the following: 1) the action of testosterone in brain development; and/or 2) competition between brain and testes for available Se when the trace element is limited.

**Aim:** Determine if MDKOSe phenotype is due to testosterone or brain Se status effect.

**Significance:** Identify potential risk factors that may need to be considered in adult hormone replacement therapies.

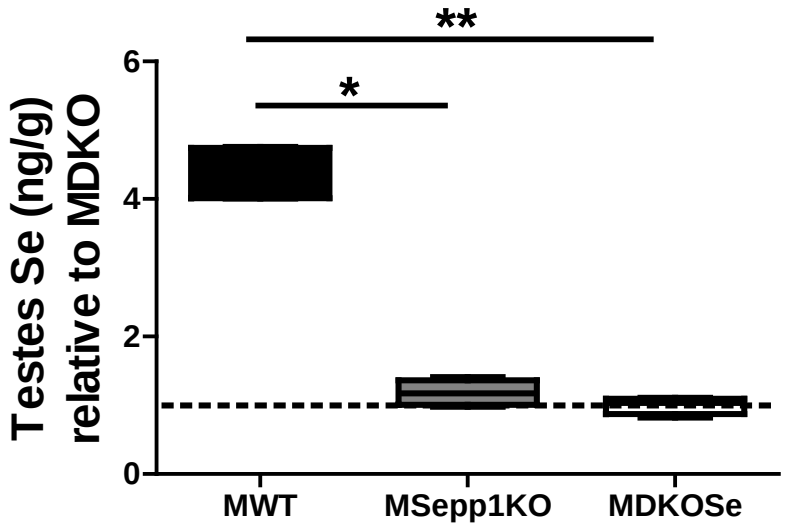



Figure 4.11: Testes Se Concentrations in Male WT, Sepp1KO, and DKOSe Mice. Mean (± SEM) Se levels relative to MDKOSe in testes of MWT (n=4), MSepp1KO (n=6) and MDKO (n=4) mice (One-way ANOVA p=0.0001, Tukey's post hoc * and ** p=<0.05).

*Preliminary group-housed mice*

Preliminary data revealed that at puberty there was a reduction in average serum the testosterone levels in MDKOSe mice compared to MWT and MSepp1KO mice, although the differences did not reach statistical significance (Figure 4.12 A). When comparing non-alpha male testosterone levels between group-housed mice, although MDKOSe were trending to lower testosterone levels then the other groups of non-alpha males this was not significant (Figure 4.12 B). No significant differences were found when comparing DKOSe males that developed audiogenic seizures and those that do not (Figure 4.12 C) but it appears that the DKOSe alpha males (males with the highest testosterone levels) fare better in retaining motor coordination at 7 weeks of age than their cage mates (Figure 4.12 D). Mice in this study were group-housed and alpha male mice were not analyzed separately, thus conclusions on the correlation between testosterone levels and neurological impairment are premature. Therefore, we conducted experiments where we re-administered testosterone to castrated and sham operated MDKOSe mice along with one group of castrated MDKOSe mice taken off of $SeH_2O$ a few days after castration, at postnatal day 37 (MDKOSe-Cast-NoSeP37).

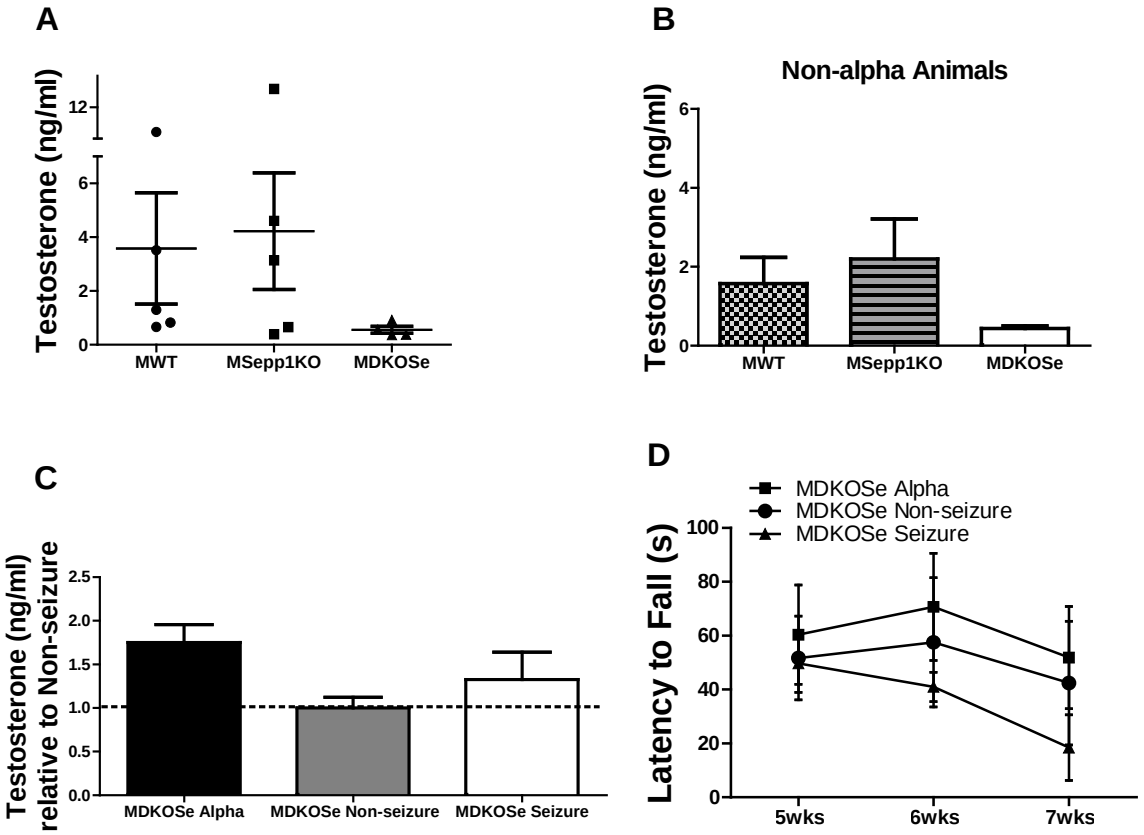



Figure 4.12: Testosterone Levels in Male WT, Sepp1KO and DKOSe Mice. A. Mean (± SEM) testosterone levels in male WT (n = 5), Sepp1KO (n = 5), and DKO (n = 4) mice; B. Mean (± SEM) testosterone levels in group housed, age matched male WT, Sepp1KO, and DKOSe males (each group consisted of 1 cage with n=4 to 5 mice); C. Mean (± SEM) testosterone levels in male DKOSe alpha (MDKOSe Alpha, mice with the highest testosterone concentration), male DKOSe mice that developed seizures (MDKOSe Seizure) and male DKOSe that did not develop seizures (MDKOSe Non-seizure) (Alpha, n=4;Non-seizure, n=6; Seizure, n=5); D. Mean (± SEM) latency to fall off Rotorod at 5, 6 and 7 weeks of age for alpha male DKOSe (MDKOSe Alpha, mice with the highest testosterone concentration), male DKOSe that did not develop seizures (MDKOSe Non-seizure) and male DKOSe mice that developed seizures (MDKOSe Seizure) (Alpha, n=4; Non-seizure, n=6; Seizure, n=5) (each group consisted of 1 cage with n=4 to 5 mice).

*Testosterone re-administration to castrated and sham-operated MDKOSe Mice*

Testosterone supplementation was able to raise the levels of testosterone in castrated and sham MDKOSe mice, with sham supplemented testosterone levels being the highest (Figure 4.13). The levels approached those seen under normal conditions. This was expected as sham mice still had the ability to produce testosterone due to intact testes in addition to the testosterone re-administration. There appears to be no change in the performance on the Rotorod nor in the Open Field tests in the castrated mice. Sham mice on testosterone supplementation had significantly better motor coordination, as tested by Rotorod, compared to the placebo group (Figure 4.14 A). There were no significant differences in locomotion average distance (Figure 4.14 B) nor average speed (Figure 4.14 C) but there was a 37% decrease in seizure occurrence in testosterone supplemented sham mice (Figure 4.14 D) indicating that testosterone was protective in this group.

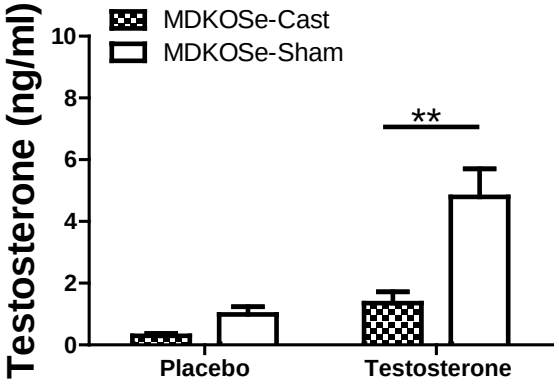



Figure 4.13: Testosterone Levels in Testosterone Supplemented MDKOSe Mice. Serum testosterone levels in testosterone and placebo supplemented castrated and sham operated MDKOSe mice (Two-way ANOVA Condition $F_{(1,22)}$=7.18, p=0.0137; Supplementation $F_{(1,22)}$=9.93, p=0.0046; Bonferroni posttests **p<0.001).

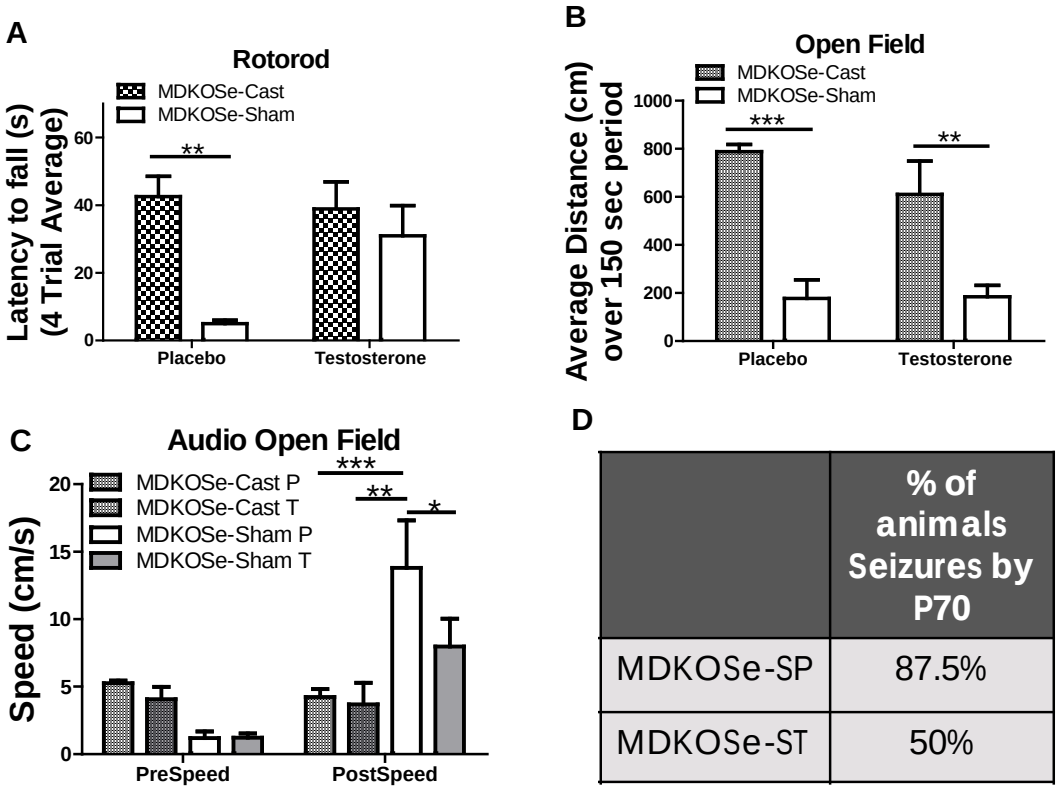



Figure 4.14: Rotorod, Open Field and Audio Open Field Behavior in Testosterone Supplemented MDKOSe Mice. Rotorod mean latency to fall (A), acclamation period average distance traveled in 2 ½ min prior to sound (B), and average speed 2 ½ min prior (PreSpeed) and 10 s after (PostSpeed) 85dB white noise played (C). D. Percentage of animals that had seizures by P70 of MDKOSe-Cast P, Cast T, MDKOSe-Sham P and Sham T mice (Two-Way ANOVA Rotorod p=0.0049; Open Field p=<0.0001; Audio Open Field p=0.0020 Bonferroni *p=<0.05; **p=<0.01; ***p=<0.001).

*$SeH_2O$ removal from castrated MDKOSe mice at P37*

When we examined the castrated group that was taken off $SeH_2O$ at P37 (MDKOSe-Cast-NoSeP37), it appeared that prepubescent castration attenuated the need for high Se supplementation in these mice. On Rotorod testing, they were able to maintain a better weekly performance than MDKOSe mice, reaching significantly better levels at 10 weeks (Figure 4.15 A) and none developed audiogenic seizures (Figure 4.15 B). Signs of reactive gliosis were not observed in the IC nor XSCP (Figure 4.16 A, B and C) although there were no significant differences in whole brain nor brain stem Gpx activity compared to MDKOSe mice (Figure 4.16

D). Together, these data indicate that the neuroprotective effect of castration stems from the elimination of the testes as a competitor for Se supply under conditions of compromised Se metabolism largely independent of any effect on testosterone levels.

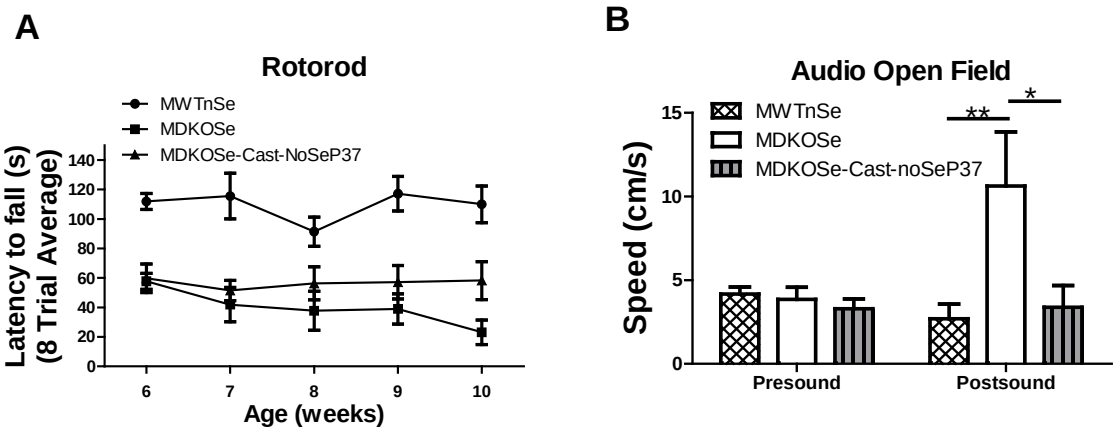



Figure 4.15: Effect of $SeH_2O$ Removal at P37 on Rotorod and Audio Open Field Behavior in Castrated MDKOSe Mice. A. Rotorod mean latency to fall from 6 to 10 weeks of age and (B) average speed 2½ minutes prior (PreSpeed) and 10 seconds after (PostSpeed) 85 dB white noise played (Two-way ANOVA: Interaction $F_{(2,50)}=3.01$ $p=0.0585$; Genotype $F_{(2,50)}=3.10$ $p=0.0537$; Sound $F_{(1,50)}=1.50$ $p=1.51$; Bonferroni post-test *$p<0.05$, **$p<0.01$) of MWTnSe, MDKOSe and MDKOSe-Cast-NoSeP37 (N=6).

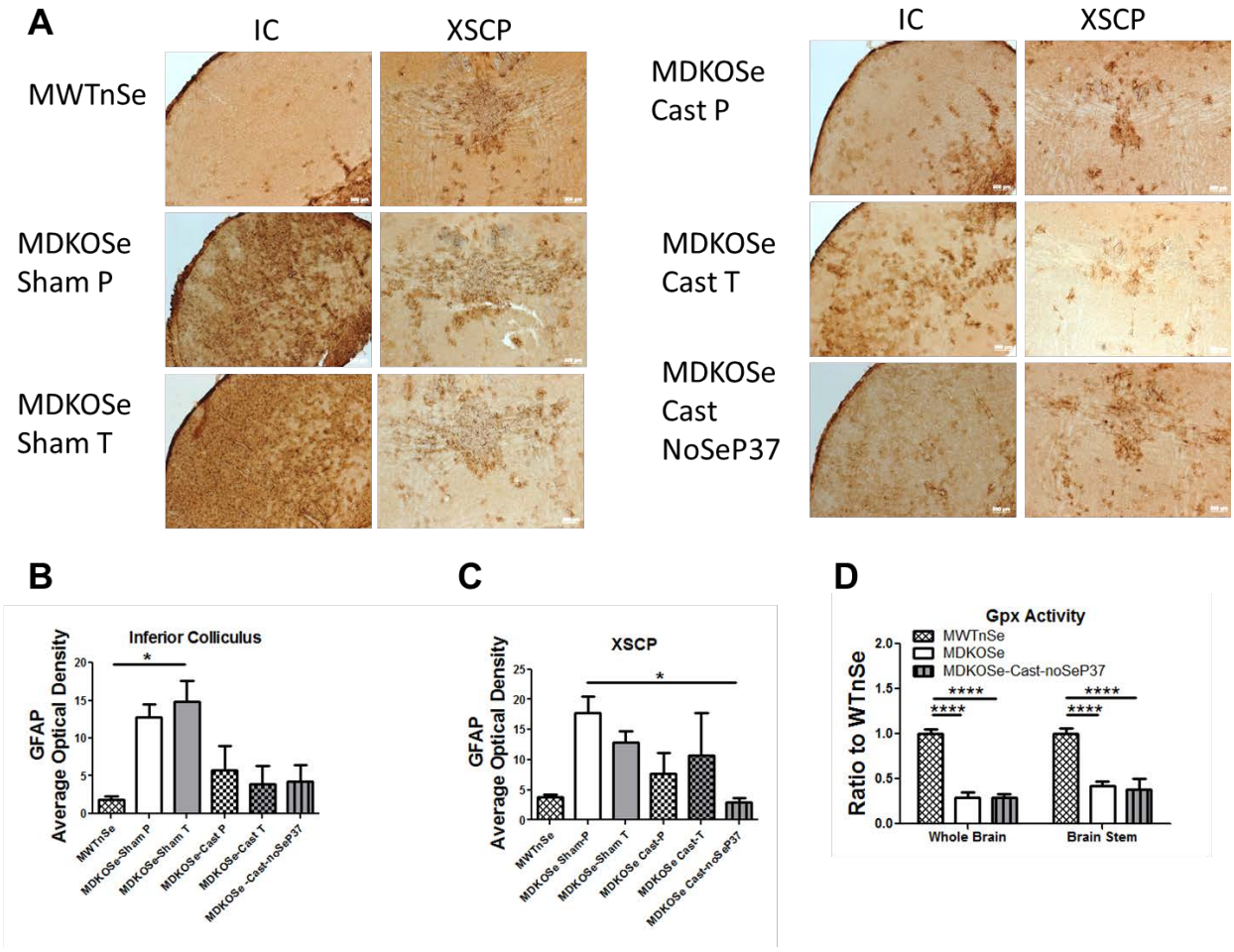



Figure 4.16: Effect of $SeH_2O$ Removal at P37 on Glial Fibrillary Acidic Protein (GFAP) Immunoreactivity and Brain Gpx Activity Levels in Castrated MDKOSe Mice. A. Select images of IC and XSCP of MWTnSe, MDKOSe-Sham P, MDKOSe-Sham T, MDKOSe-Cast P, MDKOSe-Cast T and MDKOSe-Cast-NoSeP37 mice with quantification of average optical density, IC (B) and XSCP (C) (One-way ANOVA: IC p=0.0069, Tukey's post hoc $t_{(21)}$=5.286, *p<0.05; XSCP p=0.0359, Tukey's post hoc $t_{(21)}$=4.560, *p<0.05; n=3-6 mice per group). D. Gpx Activity in the whole brain and brain stem of MWTnSe, MDKOSe and MDKOSe-Cast-NoSeP37 mice (One-way ANOVA: WB p<0.0001; BS p<0.0003; CEB p=0.0002; Tukey's Post Hoc Test ****p<0.0005, ***p<0.005; n=4-6 mice per group).

**Discussion**

Se distribution in the body varies, with priority given to the brain and endocrine tissues (91). Brain Se status appears to be independent of plasma Se status when Sepp1 is expressed in the brain (40), whereas, the lack of Sepp1 or its receptor, ApoER2, leads to the development of neurological dysfunction and deficits in spermatogenesis upon the administration of a Se-deficient diet (93; 124). When both Sepp1 and Scly are deleted, male mice exhibit decreased survival and severe neurological impairments (2). Herein, we show that castration has a neuroprotective effect in both the Sepp1KO and DKOSe phenotypes. Castration rescued the MDKOSe phenotype, delayed the neurological effects of a Se deficient diet in MSepp1KO mice and provided a partial protection from the neurotoxin, MK-801, in MWT mice. When testosterone was re-administered to castrated mice, there was no significant effect but it did appear to be beneficial to sham operated mice. This would seem to indicate that the brain and the testes compete for Se under Se deficiency thereby leading to the sex-specific phenotype of the MDKOSe mice.

Previous studies have shown that the brain has priority over the testes for Se supply. Using $^{75}$Se, it was demonstrated that in Sepp1KO mice on a Se deficient diet the Se concentration in testes decreased to about 19% of wild type (WT) whereas the brain Se decreased to about 46% of WT. When Se is re-administrated in the diet at a level 20 fold higher than the Se deficient dietary requirement of 0.1 ppm, brain levels return to normal (WT) whereas testes levels did not (84). In both the $^{75}$Se study and ours it is important to note that although the brain has priority over the testes when Se is deficient, the testes still maintain a certain level of Se even when the brain Se levels are decreased.

It is known that Se is important for sperm development as one selenoprotein, Gpx4, is incorporated as a structural component of sperm (125). It is possible that the uptake of Se from circulation to the testes for sperm development at puberty causes just enough Se to be diverted from the brain to result in the neurological phenotype. Our results seem to indicate this is the case as MDKOSe-Cast-NoSeP37 mice appear to not need the $SeH_2O$ supplementation.

The gonads have two functions: steroid hormone production, i.e. secretion of cholesterol-derived steroid hormones under control of the hypothalamo-pituitary axis (HPA), and

gametogenesis. Sex steroid hormones produced in the gonads are made from cholesterol. Cholesterol accumulates in the brain from birth to adolescence with low turnover and minimal loss in adults (126). The majority is found in myelin. It can be obtained through diet (i.e. low density lipoprotein (LDL)) or by de novo synthesis in the ER. The blood brain barrier prevents lipoprotein cholesterol uptake from circulation so the brain relies primarily on de novo synthesis. The testes, on the other hand, rely on 50% LDL uptake and 50% de novo synthesis. Ovaries rely primarily on LDL uptake (127). Free Se has been said to bind lipoproteins (e.g. very low density lipoprotein (VLDL) and LDL) which allow transport through blood and membranes via trans-membrane proteins (8). Due to the lack of the Se specific transporter Sepp1, other mechanisms of Se uptake are in effect. The ability of the testes to uptake LDL to a greater extent than the brain is just one possibility of how Se may be diverted to the testes. Investigation into alternative methods of Se transport in the absence of Sepp1 would provide further insight into this matter.

Testosterone does not reverse the effects of castration but proves to be beneficial, though not preventative, in sham MDKOSe mice. In the brains of males, testosterone is converted to estradiol and dihydrotestosterone (DHT) with actions that can be region specific (128). In aging men, brain levels of testosterone and its metabolites e.g. DHT and estradiol, decrease with age, termed andropause (129). Decreased testosterone has been indicated as a risk factor that precedes metabolic syndrome and the development of cognitive neuropathies (130; 131). Alternatively, increased testosterone has also been negatively associated with cognitive impairments in Caucasians with high oxidative stress status (132) and adverse cardiovascular events (133). In rats, testosterone deficiency leads to decreased mitochondrial gene expression and therefore decreased metabolism in the hippocampus, thus demonstrating its effect on energy metabolism through alterations in mitochondrial gene expression (123).

Reactive oxidative species (ROS) are normal byproducts of energy metabolism, and abnormal levels can lead to oxidative stress, a risk factor in neurodegeneration. We hypothesized that testosterone may contribute to the MDKOSe phenotype due to its potential effect on energy metabolism. To the contrary, this does not appear to be the case. Testosterone had no effect on castrated mice. It appears to be beneficial in sham-operated mice but its effects were not sufficient to overcome the apparent deficits due to Se being diverted from the bran to the testes.

Disruption of GABAergic inhibition seems to be the main neurological effect leading to the development of audiogenic seizures in MDKOSe mice. This is consistent with other studies demonstrating that diminished GABAergic inhibition in the inferior colliculus is associated with audiogenic seizures (134). Our findings indicate that redox imbalance impedes the development and function of $PV^+IN$. $PV^+IN$ are a subclass of GABAergic cells that synchronize activity within populations of principal neurons (135). They have high metabolic demands making them particularly susceptible to oxidative stress (136). As they mature, $PV^+IN$ are encapsulated by PNNs, a specialized extracellular matrix. PNNs are believed to limit plasticity and maintain excitatory/inhibitory balance upon neural network maturation and possibly serve as a protective barrier against oxidative stress (137; 138). In our study, the density of the $PV^+IN$ and $PV^+IN$ with PNNs appeared normal at 4 weeks of age but this was not the case at 8 weeks of age. This indicates that the maturation of $PV^+IN$ was impaired. Moreover, castration was found to promote $PV^+IN$ maturation and prevent audiogenic seizures. Together, it appears that there is a critical threshold in which decreased Se and therefore, selenoprotein levels in the brain, causes $PV^+IN$ dysfunction to occur, leading to seizure development and neurodegeneration. As a follow-up to this study, the impact on seizure threshold in the presence of varying concentrations of GABA agonist/modulators could be used to further probe the functional GABAergic defects in the DKOSe mice.

**Conclusion**

MDKOSe mice exhibited severe neurological dysfunction and neurodegeneration in brain nuclei and tracts involved in auditory and motor pathways, with GABAergic system defects in the IC (2). Preadolescent castration rescued the MDKOSe phenotype and eliminated the need for $SeH_2O$ supplementation in MDKOSe adults. It delayed the onset of neuromotor deficits in MSepp1KO mice on a Se deficient diet and was protective of MWT mice exposed to MK-801 neurotoxin. Testosterone re-administration to sham-operated mice appeared to be beneficial but not preventative. Together, these results suggest that there is a critical time frame in which selenium is essential for the prevention of adolescent and adult neurological impairment in males and that males may have a greater need than females for Se during the course of their sexual reproductive period due to the need for selenium in the testes.

# Chapter 5: Conclusions and Future Directions – potential model for the study of ictogenesis and novel drug testing

## Introduction

Seizures can spontaneously develop in both male and female DKOSe mice. This spontaneous development is due to a nutritional deficiency caused by a deficit in the metabolism of the micronutrient Se, and it does not affected all mice equally e.g. 75% of males and 83% of females by ten to twelve weeks of age. This provides three key areas for the study of seizure development: spontaneous seizure occurrence, nutritional influence, and influence of sex.

Our results indicate that there is a critical time point in which Se is essential for the prevention of neurological impairment in both male and female DKOSe mice. MDKOSe may have a greater need for Se during the course of their sexual reproductive period due to competition between the testes and the brain for Se. Disruption of the maturation of GABAergic inhibition seems to be the main neurological effect leading to the development of audiogenic seizures in MDKOSe mice which can be rescued with preadolescent castration. Castration delays onset of neuromotor deficits in MSepp1KO mice on a Se deficient diet and is protective of MWT mice exposed to the neurotoxin dizocilpine maleate (MK-801). Testosterone re-administration to sham-operated MDKOSe mice appears to be beneficial but not preventative.

The specific deficits in behavioral tests and underlying GABAergic system maturation disruption could allow DKOSe mice to potentially serve as a model for the study of ictogenesis. Further investigation into the roles of selenoproteins at key points of development would be beneficial in determining dietary supplementation requirements. This knowledge can then be used to optimize individualized nutrition in both humans and animals, thereby having potential application to preventing disease pathology in the case of Se deficiency and as a potential model for targeted therapeutic agents in epileptic patients.

## Seizures

Seizure disorders are considered a major type of neurological diseases (139). Typically, we think of seizures as convulsions where the body shakes uncontrollably, but this is only a symptom of some types of seizures. A seizure, as defined by Medline Plus, is “the physical

findings or changes in behavior that occur after an episode of abnormal electrical activity in the brain" (140). This can range from a brief, 1 second action, to prolonged 1 to 2 minute actions. Convulsions associated with seizures depend on the area of the brain where abnormal activity occurs and if it spreads to other areas or remains focal.

Seizures have been categorized into several types. Generalized seizures normally begin in both hemispheres of the brain. These include tonic-clonic seizures (grand mal seizures) where there is a stiffening phase followed by a jerking phase which can last up to two minutes, and absence (petit mal) seizures involving brief periods of loss of consciousness followed by returning to normal cognition. Sometimes they are accompanied by repetitive blinking and rolling of the eyes. Myoclonic seizures that last only 1 to 2 seconds and may include a jerk or loss of consciousness but are difficult to identify due to the brevity of the seizure, and atonic seizures where there is a sudden loss of muscle tone leading to a falling or "dropping" of the person (141).

Partial seizures (focal seizures) normally start in a specific area of the brain and may remain limited to that area or spread to other areas of the brain. If they spread to the other hemisphere of the brain then they may lead to a secondary generalized seizure as described above. These are the most common type of seizures found in adults with epilepsy (142; 143). They can involve any movement, sensory or emotional symptoms depending on which area of the brain that is involved. These seizures can be "simple" where they last only 30 to 60 seconds with consciousness retained or "complex" lasting 1 to 2 minutes with impairment or loss of consciousness.

Other seizure types include gelastic and dacrystic seizures. These seizures are commonly overlooked due to their unusual manifestations (144). Gelastic seizures manifest as bouts of uncontrollable laughter and/or giggling. They cause facial contractions that cause the person to look like they are smiling. Dacrystic is when the vocalization sounds like crying and the facial contractions cause the person to look like they are frowning. These are commonly overlooked in children unless other clinical signs (auras) that occur prior to seizure occurrence, such as eye dilation, are recognized. Usually these seizures are a pre-development of other types of seizures such as absence, complex partial or generalized seizures. These types of seizures may be triggered by hypothalamic hamartomas (benign tumor of hypothalamus). If detected early, therapy can be administered to reduce the tumor size to avoid further progression.

Non-epileptic seizures (NES) are not caused by a central nervous system (brain) abnormality (145). They consist of two types, organic or psychogenic. Organic NESs are physical in nature, e.g. low blood sugar or changes in heart function. Their symptoms can be syncope (fainting) or symptoms similar to one of the other seizures mentioned above. Normally, once the underlying cause is corrected the seizures can be controlled or stopped.

Psychogenic NESs contain a mental health element such as emotional stress or other anxieties. These include traditional seizure type symptoms and panic attacks. They are divided into dissociative (involuntary) or factitious (under conscious control). Regardless of their causes, they are normally "sudden, short and cause a change of person's awareness" (145). Behavior modification therapies are normally used in treatment of these types of seizures.

As mentioned above, seizures can be a one-time occurrence or epileptic in nature (e.g. epilepsy). According to the World Health Organization, epilepsy affects about 50 million people worldwide and six cases out of ten have no known cause (146). Those with a known cause are sometimes considered secondary epilepsy because the seizure is secondary to a primary cause such as brain damage and/or malformation; brain trauma due to stroke or blunt trauma; tumor or genetic defect.

The most common type of epilepsy is temporal lobe epilepsy (TLE) (142). TLE is associated with the medial temporal lobe regions and patients with this type of epilepsy normally do not respond to clinical treatment (147). The temporal lobe is known to be involved in visual memory retention, sensory input processing, language and emotion together with the medial temporal lobe containing the amygdala, brainstem, hippocampus and neocortical regions. Since most patients do not respond to drug treatment, surgery is usually used to remove the affected brain areas.

Currently, it is believed that 70% of patients with epilepsy will respond to treatment (146). Treatments include antiepileptic drugs which, if taken regularly, have been shown to prevent seizures in 50% of patients and reduce seizure activity in another 20% (148). For some patients, after taking antiepileptic drugs for several years they can be weaned off to be seizure free without medication. For those that do not respond to the antiepileptic drugs, other therapies such as electrical/magnetic stimulation, ketogenic diet, surgery and behavioral modification can be implemented with or without antiepileptic medications.

Electrical/magnetic stimulation is where electrical or magnetic pulses are used to stimulate brain activity. Though the current studies using this method seem to be focused more on mental disorders such as severe depression, it has been found to help some people with epilepsy. Vagus nerve stimulation, wherein a device is implanted that sends 30 second pulses through the left vagus nerve, can alter certain neurotransmitters in the areas of the brain that affect mood and sleep as well as stimulating the major organs such as heart, lungs and intestines (149).

Diet is also used for treatment of some types of seizures. The link between nutrition and epilepsy has yet to be elucidated completely and is complicated by the possibility of multiple unknown causes (150). Some people are able to control seizure by fasting (151). It has now been found that about 1/3 of children placed on a ketogenic diet become seizure free. The ketogenic diet mimics starvation forcing the body to burn fat as its main source of energy. Children are normally placed on this diet and weaned off antiepileptic drugs over a two-year period. If successful, the child can then be returned to a normal diet, seizure free or with successful medication control.

Surgery is normally used as a last resort when the individual does not respond to available treatments and the seizure arises from a focal area that can be specifically identified and removed. Like all treatments, surgery does not work for some patients as the source of the seizures may migrate to the opposite lobe which cannot be removed without significantly altering the patient's mental capacity.

Behavioral modification therapies have been used by many people with mixed results. They are considered complementary approach as they may help some people improve their quality of life (152). This type of approach is used most often in NES patients wherethe cause is voluntary or learned, and can be reversed by changes in mental thought processes.

Overall, people who have epilepsy, if given access to treatment therapy, have a good chance (70%) of controlling their seizures. But there is still a need for improvement in current therapies and to find new therapies for those that do not respond to existing treatments. When setting up a study that explores treatment options, current models use one of two hypotheses, recurrent excitation, which assumes hyper excitability due to abnormal excitatory circuitry, or inhibition, the loss of inhibitory neurons. From these hypotheses, researchers have developed two types of animal models, post-status epilepticus (severe insult model due to chemo-

convulsant, electrical stimulation, etc.) and kindling models (repetitive exposure to mild electrical stimulation leading to seizure development). With these models, researchers have been looking at new potential therapies such as cell therapy (cell grafts), neuroprotective agents and other biological agents that might help to restore normal function of the affected areas.

**Animal Models in Basic Research**

In basic research, the application of the knowledge obtained is not always readily apparent. It may take years of successive studies, sometimes from multiple independent labs, before basic research leads to beneficial treatments that can be applied in clinical settings. Part of this process involves the use of animal models. By using animal models one can study molecular pathways that contribute to diseaseoutcomes and pathology *in vivo*. Targets of cellular mechanisms can then be investigated to determine if a modification (e.g. administration of a novel drug, gene editing or surgery) can be used as a therapy without causing adverse side effects.

The mouse (*Mus musculus*) has been used as the animal model in basic research for many human diseases. Mice are mammals with a genome of approximately 30,000-40,000 genes, 99% of which have human homologues (153). Mice have a short gestation period (about a month) and relatively short lifespan (1-2 years) which make them ideal for studying development, puberty and some properties of aging in a reasonable timeframe. There are multiple resources available that help make mouse-human comparisons including Translating Time (http://www.translatingtime.net/) and the Allan Brain Atlas (http://www.brain-map.org/). In addition, due to advances in gene manipulation techniques, “custom” mice are becoming more common and can be bred to investigate the function of specific genes.

An area of medical research that has made advances using animal models is the study of potential treatments for epilepsy and seizure disorders. The brain is a dynamic environment involving a complex interaction of multiple molecular and cellular components which cannot be completely duplicated by current *in vitro* techniques. For such a dynamic system, mouse models have been useful tools. Detailed reviews by Loscher (154), Auvin (155) and Grone and Baraban (156) discuss the current models used for research in this area. In general, these models fall into two categories, induced or genetic. Induced models require the administration of external stimuli or insult to normal mice allowing researchers to produce both acute and chronic seizure

disorders. The development of genetically altered animals have allowed for the modeling of known epileptic genes (156).

But, like models of other diseases, currently there is no single model that has been able to completely represent all features of human seizure disorders. One of the limitations arising from induced models is that most human epileptics have multiple spontaneous seizures indicating that the ictogenesis arises possibly from a different mechanism than the one produced by electrical or chemical induction. Genetic models give insights to inheritable phenotypes but do not necessarily address all aspects of the condition or epilepsy of idiopathic origin.

When interpreting animal studies, investigators must keep in mind that most animal models do not develop all of the characteristics found in human epileptic seizures (157; 158; 159). This is complicated by the fact that normally the cause of the human condition is not fully known and therefore, it is unknown how much or little the animal model mimics the clinical condition. For these reasons it might be better to think of animal models as partial or "incomplete" models. They can be used to investigate some of the disease mechanism thereby elucidating specific parts of the disease process. The more one is familiar with the aspects of the disease state in the animal model the easier it will be to interpret the experimental results and develop relevant treatments.

Even with incomplete models, progress has been made to develop treatments for patients with seizure disorders. The current models have led to the development of anticonvulsant drugs to which 50% of patients respond positively and seizure reduction is seen in another 20% (148). Room for improvement is still evident as there are still no treatment options for 30% of patients and 20% of those on active treatment still suffer from some type of seizure activity. Continued basic research on the ictogenesis as well as the mechanisms by which anticonvulsants may be able to suppress seizure activity is needed. This will lead to the enhancement of experimental interpretation and finally better treatment options to those who suffer from seizure episodes.

**Selenium and epilepsy**

Selenium has become increasing thought to play a role in the pathologenesis of epilepsy due to the role of selenoproteins in defense against oxidative stress. In animal models, oxidative stress imbalance has been shown to be attenuated upon selenium supplementation (160; 161; 162; 163; 164; 82) with selenium deficiency leading to increased seizure susceptibility (2; 70;

165). Patients with epilepsy have been found to have oxidative stress abnormalities such as decreased antioxidants and increased lipid peroxidation (166). Studies of febrile seizures in children have indicated decreased selenium serum levels (167; 168; 169; 10; 11) with some studies showing an attenuation of seizure activity upon selenium supplementation (9).

**Sepp1 and Scly Double Knockout: potential model for studying seizure development and epilepsy**

As stated earlier, six out of ten patients with epileptic seizures have no known cause and most have multiple spontaneous seizures. Two hypotheses for the causes of epilepsy are abnormal excitatory or inhibition circuitry. Induced and genetic animal models of seizures have been developed to study these aspects but still 30% of patients do not respond to current therapies. In DKOSe mice, both male and female mice develop seizures and therefore they may serve as a potential animal model for studying seizure genesis and for therapeutic drug testing.

In compiling all our data to date, we have the following information that can be used for investigational purposes:

Behavioral (Figure 5.1) and body composition (Figure 5.2): Motor cordination is expected to decrease as DKOSe mice age, with a percentage of mice in each sex that will develop seizures by P70. For MDKOSe mice, the percentage that will develop seizures is about 73-77% between 8 to 12 weeks of age with approximately 40% requiring euthanasia prior to 10 weeks of age. Decrease in motor coordination begins at about 6 weeks of age. This is accompained by a wobbly or abnormal gait. Weight loss along with decreasing food and water intake will commence approximately a week prior to seizure development. Aura and ictal stages are of consistent duration with the postictal stage duration lengthening as the condition of the mice deteriorates. Castration appears to attenuate the need for $SeH_2O$ supplementation after age P37, indicating the testes ability to divert Se from the brain.

In FDKOSe mice, decreases in motor coordination starts at 7 weeks of age. This becomes significantly different from FWTnSe mice by 10 weeks of age and is accompanied by significant weight gain. When $SeH_2O$ supplementation is removed at weaning (P22) about 83% will develop a neurological phenotype by 10 weeks of age. Removal of $SeH_2O$ supplementation at adulthood (P37) appears to not lead to seizure development but it is still unknown whether this would affect them in other aspects e.g. fertility. As the FDKOSe mice have not been as

extensively studied as the MDKOSe mice, further and more comprehensive studies on parameters such as food and water intake, weight change and signs leading up to seizure development in FDKOSe-NoSeP22 need to be pursued.

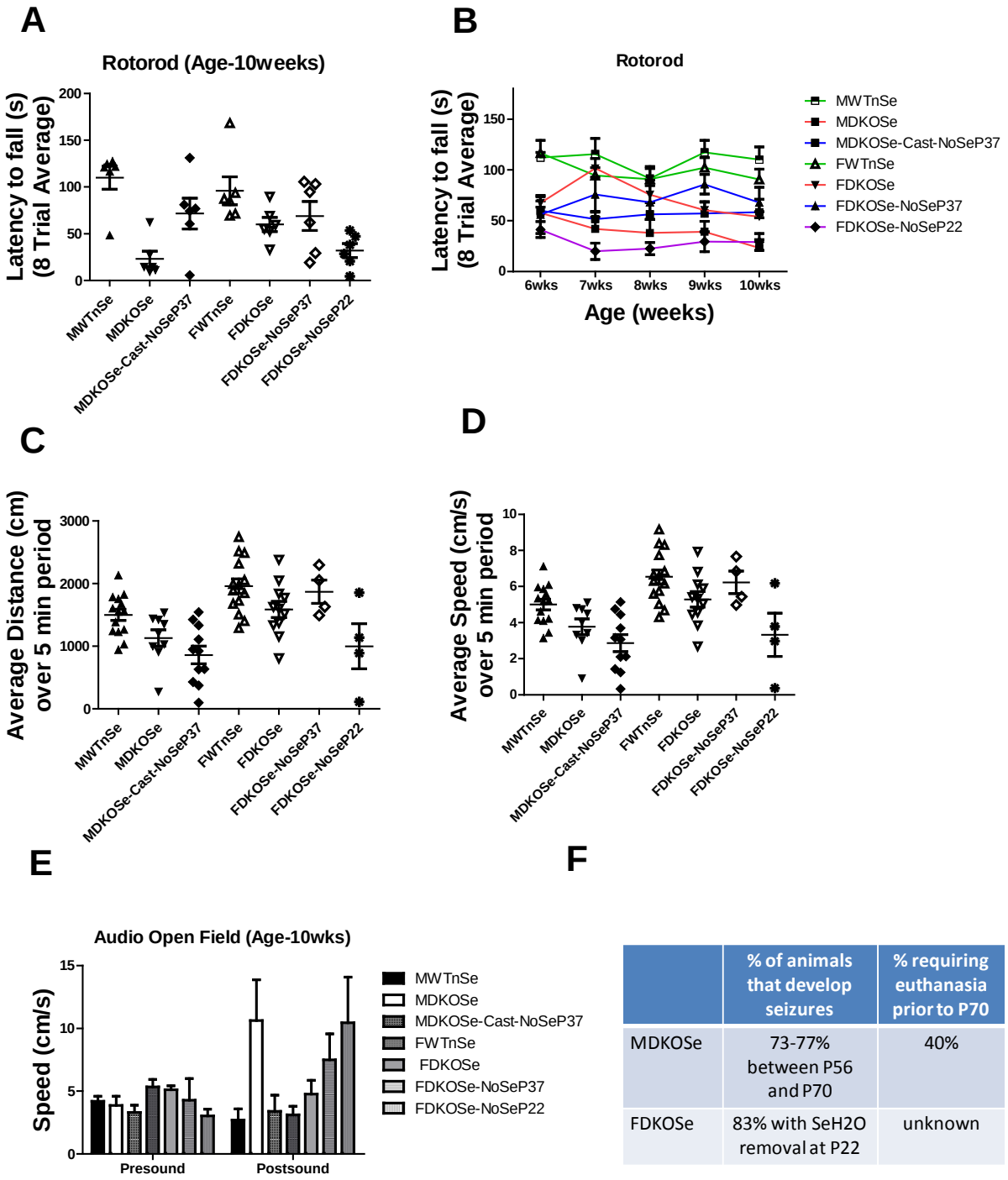
Brain regions: Specific brain areas in the male and female DKOSe mice that show signs of reactive gliosis and neurodegeneration are the inferior colliculus (IC) and decussation of superior cerebellar peduncles (XSCP). The IC is the principal midbrain nucleus of the auditory pathway involved in auditory information processing and sound localization. The XSCP is where motor tract fibers from cerebellar nuclei decussate in the caudal midbrain before terminating in the contralateral red nucleus. Reactive gliosis can be detected as early as 4weeks of age in the IC of MDKOSe and neurodegeneration in the IC and XSCP by 10 weeks of age. FDKOSe-NoSeP22 mice show reactive gliosis in both the IC and XSCP by 10 weeks of age with significant neurodegeneration in the XSCP.

Brain inhibitory network: In the brain of DKOSe mice, the brain GABAergic system (inhibitory network) has maturation defects. MDKOSe mice have decreased glutamate decarbosylase ($GAD_{67}$), the enzyme that catalyzes decarboxylation of glutamate to gamma-amino butyric acid (GABA), which is the most common inhibitory transmitter in the mammalion nervous system. These mice also have decreased density of parvalbumin expressing interneurons ($PV^{+}IN$) (2). PV is a calcium binding protein found on a subpopulation of GABAergic cells. $PV^{+}IN$ are fast spiking interneurons which control output of principal neurons. PV+IN are necessary for fast rhythmic neuronal synchrony and for facilitation of information processing during cognitive tasks.

As $PV^{+}IN$ mature they become encased by specialized extracellular matrix or agrecan-enriched perineuronal nets (PNNs) which promote their maturation and synaptic and network stability (137). This maturation process appears to be disrupted in MDKOSe mice, and this disruption is preceded by reactive gliosis. FDKOSe-NoSeP22 mice show reactive gliosis and neurodegeneration in the same brain areas as MDKOSe mice which indicates that the same neural processes are affected in both males and females. Further studies investigating the GABAergic deficits involved in seizure development would be beneficial in identifying this.

## Conclusion

The aforementioned provides investigational targets for future studies in DKOSe male and female mice. Specific behavioral deficits e.g. motor coordination and weight change, occur before seizure onset, allowing deterioration progression to be monitored over a period of time. Deficits in GABAergic system maturation in areas of the brain involved in motor coordination and auditory processing appear to be the underlying contributory factors. $SeH_2O$ supplementation appears to be required during development and adolescent periods but can be removed in FDKOSe and castrated MDKOSe mice after P37. Combined, DKOSe mice could potentially serve as a model for the study of ictogenesis and therapeutic drug testing of novel drugs for epileptic patients in cases of malnutrition, and to elucidate the compensatory mechanisms that are distinct between males and females during development.




| | % of animals that develop seizures | % requiring euthanasia prior to P70 |
|---|---|---|
| MDKOSe | 73-77% between P56 and P70 | 40% |
| FDKOSe | 83% with SeH2O removal at P22 | unknown |

Figure 5.1: Combined Motor Coordination, Locomotion and Seizure Occurrence Data. Mean latency to fall at ten weeks of age (A), mean latency to fall from six to ten weeks of age (B), distance travel (C) and average speed travel (D) in 5 minute period of open field, and speed prior to and 10 seconds immediately after start of 85 dB white noise (E) of MWTnSe, MDKOSe,

MDKOSe-Cast-NoSeP37, FWTnSe, FDKOSe, FDKOSe-NoSeP37 and FDKOSe-NoSeP22 mice. F. Table of percentage of animals that develop seizures by 10 weeks of age.

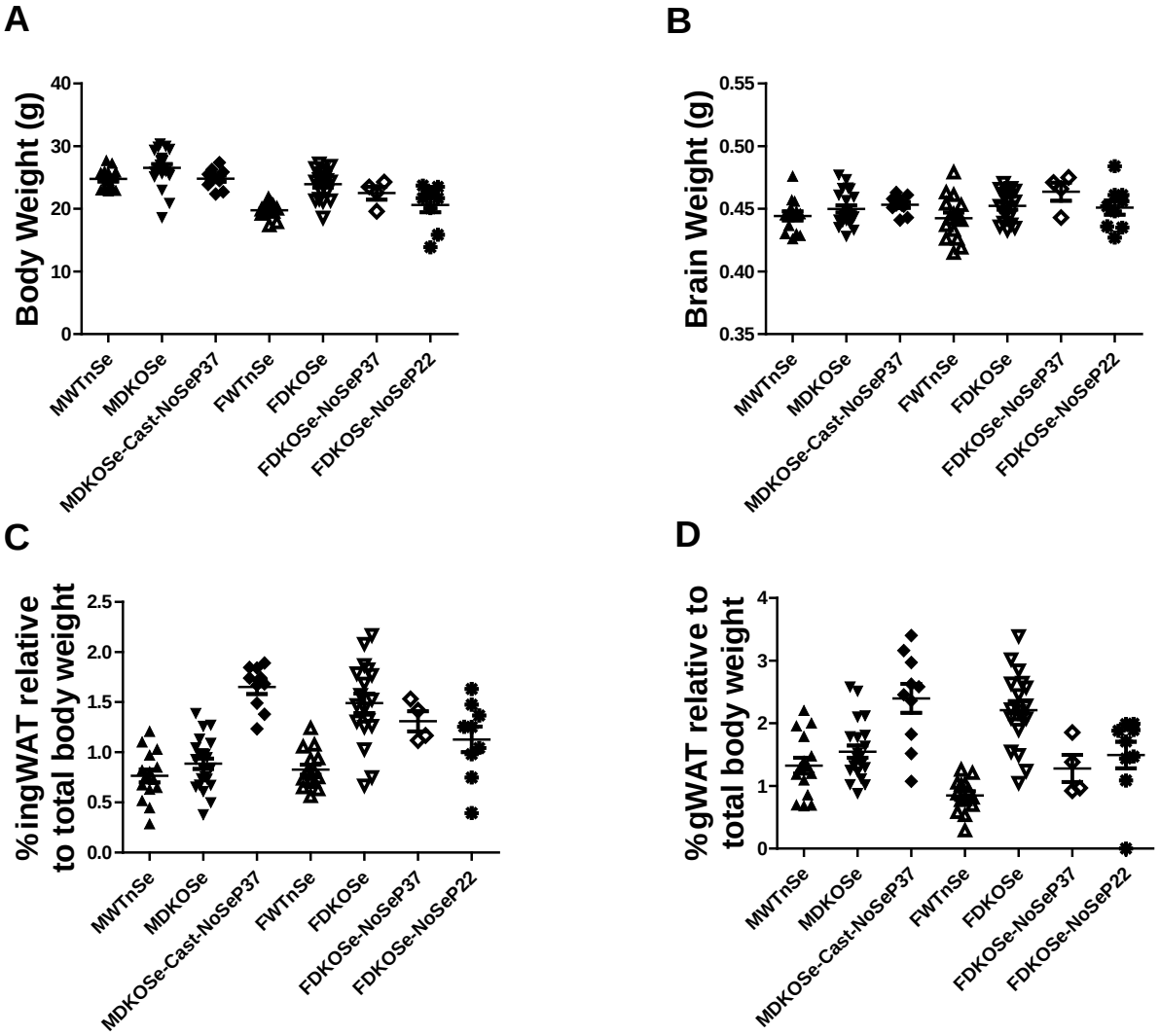



Figure 5.2: Combined Body Composition Data. Body (A), brain (B), %ingWAT (C), and %gWAT (D) of MWTnSe, MDKOSe, MDKOSe-Cast-NoSeP37, FWTnSe, FDKOSe, FDKOSe-NoSeP37 and FDKOSe-NoSeP22 mice.

# List of Publications

1. Pitts MW*, Kremer PM*, Hashimoto AC, Torres D, Byrns CN, Williams C, and Berry MJ. (2015). Competition between the Brain and Testes under Selenium-Compromised Conditions: Insight into Sex Differences in Selenium Metabolism and Risk of Neurodevelopmental Disease. The Journal of Neuroscience, 18 November 2015, 35(46):15326-15338; doi:10.1523/JNEUROSCI.2724-15.2015, *Co-first authors.
2. Pitts MW, Byrns CN, Ogawa-Wong AN, Kremer P, Berry MJ. (2014) Selenoproteins in nervous system development and function. Biol Trace Elem Res. 2014 Dec;161(3):231-45. doi: 10.1007/s12011-014-0060-2
3. Pitts MW, Reeves MA, Hashimoto AC, Ogawa A, Kremer P, Seale LA, and Berry MJ. (2013) Deletion of Selenoprotein M Leads to Obesity Without Cognitive Deficits, J. Biol. Chem. 2013 Sep 6; 288(36):26121-34. jbc. M113.471235.

# References

1. Fairweather-Tait, S. J., Bao, Y., Broadley, M. R., Collings, R., Ford, D., Hesketh, J. E., et al. Selenium in human health and disease. *Antioxidants and Redox Signaling.* 2011, 14:1337-1383.

2. Byrns, C. N., Pitts, M. W., Gilman, C. A., Hashimoto, A. C., & Berry, M. J. Mice lacking selenoprotein P and selenocystein lyase exhibit severe neurological dysfunction, nerodegeneration, and audiogenic seizures. *The Journal of Bilogical Chemistry.* 2014. 289; 14: 9662–9674.

3. Oldfield, J.E. A brief history of selenium research: From alkali diseas to prostate cancer (from poison to prevention). *American Society of Animal Science.* 2002, [online] https://www.asas.org/docs/publications/oldfieldhist.pdf?sfvrsn=0 [accessed Feb 6, 2015]

4. Christesen, K. Selenium - the Essential Trace Mineral. *The Biology of the Goat.* [Online] 2006. [Cited: Jul 18, 2014.] http://www.goatbiology.com/selenium.html.

5. Schwarz, K., & Foltz, C. M. Selenium as an integral part of factor 3 against dietary necrotic liver degeneration. *Nutrition Classics. Journal of the American Chemical Society.* 1957, 79:3292-3293.

6. McCoy, K. E., & Weswig, P. H. Some selenium responses in the rat not related to vitamin E. *Journal of Nutrition.* 1969, 98:383-389.

7. WHO. Trace elements and human nutrition. [Online] 1996.

8. Mehdi, Y., Hornick, J.-L., Istasse, L., & Dufrasne, I Selenium in the environment, metabolism and involvement in body functions. *Molecules.* 2013, 18:3292-3311.

9. Weber, G. G., Maertens, P., Meng, X., & Pippenger, C. E. Glutathione peroxidase deficiency and childhood seizures. *Lancet.* 1991, 337:1443-44.

10. Amiri, M., Farzin, L., Moassesi, M. E., & Sajadi, F. Serum trace element levels in febrile convulsion. 2010, 135:38-44.

11. Mahyar, A., Ayazi, P., Fallahi, M., & Javadi, A. Correlation between serum selenium level and febrile seizures. *Pediatric Neurology.* 2010, 43:331-334.

12. Schweizer, U., Dehina, N. and Schomburg, L. Disorders of selenium metabolism and selenoprotein function. *Current Opinion in Pediatrics.* 2011, 23:429-435.

13. Gupta, M and Gupta, S. An Overview of Selenium Uptake, Metabolism, and Toxicity in Plants. *Front Plant Sci.* 2017, Jan 11; 7:2074.

14. El Mehdawi, A.F., Quinn, C.F. and Pilon-Smits, E.A. Effects of se hyperaccumulation on plant-plant interactions: evidence for elemental allelopathy? *New Phytol.* 2011, Jul;191(1):120-31.

15. Blazina, T., Sun, Y., Voegelin, A., Lenz, M., Berg, M., & Winkel, L. Terrestrial selenium distribution in China is potentially linked to monsoonal climate. *Nat Commun.* 2014, Sept. 2; 5:4717.

16. Combs, G.F. Jr. Selenium in global food systems. *Br J Nutr.* 2001, May; 85(5):517-47.

17. Jones, G., Droz, B., Greveb, P., Gottschlkc, P., D, P., Mcgrathe, S., et al. Selenium deficiency risk predicted to increase under future climate change. *PNAS.* 2017, 114(11):2848–2853.

18. FDA. Minerals. [Online] 2017. www.fda.gov/nutritioneducation.

19. National Institute of Health. Selenium: Dietary Fact Sheet. *NIH.* [Online] 2016. [Cited: 5 8, 2017.] https://ods.od.nih.gov/factsheets/Selenium-HealthProfessional/ .

20. National Institute of Health. Office of Dietary Supplements. *Selenium. Sietary Supplement Fact Sheet.* [Online]http://ods.od.nih.gov/factsheets/Selenium-HealthProfessional/[accessed Feb. 6, 2015] : USA.gov, Jul 2, 2013.

21. Fenech, M., El-Sohemy, A., Cahill, L., Ferguson, L., French, T., Shyong Tai, E., et al. Nutrigenetics and Nutrigenomics: Viewpoints on the Current Status and Applications in Nutrition Research and Practice. *Journal of Nutrigenetics and Nutrigenomics.* 2011, 4:69-89.

22. Georgieff, M.K. Nutrition and the developing brain: nutrient priorities and measurements. *The American Journal of Clinical Nutrition.* 2007, 85(suppl):614S-20S.

23. Dobbing, J. Infant nutrition and later achievement. *The American Journal of Clinical Nutrition.* Feb 1985, 41:477-484.

24. Roman, G.C. Nutritional disorders in tropical neurology. [ed.] H.H. Garcia, H.B. Tanowitz and O.H. Del Brutto. *Handbook of Clinical Neurology.* 2013, 381-404.

25. FAO, United Nations. [Online] 2004.

26. Food and Agriculture Organization of the United Nations. The Spectrum of Malnutrition. *http://www.fao.org.* [Online] [Cited: June 27, 2017.] http://www.fao.org/worldfoodsummit/english/fsheets/malnutrition.pdf.

27. WHO. Global Health Estimates. [Online] 2015.

28. Ames, B.N. Low micronutrient accelerate may accelerate the degenerativediseases of aging through allocation of scarce micronutrients by triage. *PNAS.* 2006, Nov 103(47):17589–17594.

29. Neibergs, H.L and Johnson, KA. Alpharma Beef Cattle Nutrition Symposium: nutrition and the genome. *Anim Sci.* 2012, 90:2308–2316.

30. Bull, C and Fenech, M. Genome-health nutrigenomics and nutrigenetics: nutritional requirements or 'nutriomes' for chromosomal stability and telomere maintenance at the individual level. *Proc Nutr Soc.* 2008, May, 67(2):146-56.

31. Fenech, M.F. Dietary reference values of individual micronutrients and nutriomes for genome damage prevention: current status and a road map to the future. *Am J Cln Nutr.* 2010, May, 91(5):1438S-1454S.

32. Deb, R., Chakraborty, S., Mahima, Verma, A., Tiwari, R., & Dhama, K. Nutrigenomics and its role in male puberty of cattle: a mini review. *Pak J Biol Sci.* 2014, Feb 1;17(3):329-34.

33. Burk, R.F., Hill, K.E and Motley, A.K. Plasma selenium in specific and non-specific forms. *BioFactors.* 2001, 14:107-114.

34. Behne, D. and Kyriakopoulos, A. Mammalian selenium-containing proteins. *Annu. Re. Nutr.* 2001, 21:453-73, pp. 21:453-73.

35. Mickiewicz, B., Villemaire, M., Sandercock, L., Jirik, F., & Vogel, H. Metabolic changes associated with selenium deficiency in mice. *BioMetals.* 2014, Dec; 27(6):1137-1147.

36. Locasale, J.W. Serine, glycine and the one-carbon cycle: cancer metabolism in fulll circle. *Nat Rev Cancer.* 2013, Aug;13(8):572-583.

37. Davis, C.D. and Uthus, E.O. Dietary Folate and Selenium Affect Dimethylhydrazine-Induced Aberrant Crypt Formation, Global DNA Methylation and One-Carbon Metabolism in Rats. *The Journal of Nutrition.* 2003, Sept; 133(9):2907-2914.

38. Metes-Kosik, N., Luptak, I., Dibello, P., Handy, D., Tang, S., Zhi, H., et al. Both Selenium Deficiency and Modest Selenium Supplementation Lead to Myocardial Fibrosis in Mice via Effects on Redox-Methylation Balance. *Mol Nutr Food Res.* 2012, Dec; 56(12):1812-1824.

39. Young, S.N. The Significance of Tryptophan, Phenylalanie, Tyrosie, and Their metabolites in the nervous system. [book auth.] Ed. Abel Lajtha. *Hanbook of Neurochemistyr, Vol 3 (2nd Ed.).* Montreal Quebec : Plenum Publishing Corporation, 1983, pp. 559-581.

40. Schweizer, U., Streckfub, F., Pelt, P., Carlson, B. A., Hatfield, D. L., Kohrle, J., et al. Hepatically derived selenoprotein P is a key factor for kidney but not for brain selenium supply. *Journal of Biochem.* 2005, 386:221-226.

41. Burk, R. F. and Hill, K.E. Selenoprotein P - expression, functions, and roles in mammals. *Biochimica et Biophysica.* 2009, 1790:1441-1447.

42. Casey, C., Guthrie, B., Friend, G., & Robinson, M. Selenium in human tissues from New Zealand. *Arch Environ Health.* 1982, May-Jun; 37(3):133-5.

43. Oster, O, Schmiedel, G and Prellwitz, W. The organ distribution of selenium in German adults. *Biol Trace Elem Res.* 1988, Jan-Apr;15:23-45.

44. Zachara, B., Pawluk, H., Bloch-Boguslawska, E., Sliwka, K., Korenkiewicz, J., Skok, Z., et al. Tissue level, distribution, and total body selenium content in healthy and diseased humans in Poland. *Arch Environ Health.* 2001, Sept-Oct; 56(5):461-6.

45. Chen, J. and Berry, M.J. Selenium and selenoproteins in the brain and brain diseases. *J Neurochem.* 2003, Jul; 86(1):1-12.

46. Behne, D and Wolters, W. Distribution of selenium and glutathione peroxidase in the rat. *J Nutr.* 1983, Feb;113(2):456-61.

47. Hefnawy, A.E. and Tortora-Perez, J. The importance of selenium and the effects of its deficiency in animal health. *Small Ruminant Research.* 2010, 89:185-192.

48. Rayman, M.P. Selenium and human health. *Lancet.* 2012, Mar 31; 379(9822):1256-68.

49. Steinbrenner, H and Sies, H. Selenium homeostasis and antioxidant selenoproteins in brain: implications for disorders in the central nervous system. *Arch Biochem Biophys.* 2013, Aug 15; 536(2):152-7.

50. Kühbacher, M., Bartel, J., Hoppe, B., Alber, D., Bukalis, G., Bräuer, A., et al. The brain selenoproteome: priorities in the hierarchy and different levels of selenium homeostasis in the brain of selenium-deficient rats. *J Neurochem.* 2009, Jul;110(1):133-42.

51. Prohaska, J.R. and Ganther, H.E. Selenium and glutathione peroxidase in developing rat brain. *J Neurochem.* 1976, Dec; 27(6):1379-87.

52. Hoppe, B., Bräuer, A., Kühbacher, M., Savaskan, N., Behne, D., & Kyriakopoulos, A. Biochemical analysis of selenoprotein expression in brain cell lines and in distinct brain regions. *Cell Tissue Res.* 2008, Jun; 332(3):403-14.

53. Arthur and Becket 2000, edited by A.-M. Roussel, Richard Allen Anderson, Alain Favier,. Trace elements in man and animals 10, some biochemical functions of selenium in animals and man. s.l.:klumer academic/plenum publishers, New York 2000.

54. Arner, E.S. Selenoproteins-what unique properties can arise with selenocysteine in place of cysteine? *Exp Cell Res.* . 2010 May 1;316(8):1296-1303. doi: 10.1016/j.yexcr.2010.02.032.

55. Darras, V.M., Houbrechts, A.M. and Van Herck, S.L.J. Intracellular thyroid hormone metabolism as a local regulator of nuclearthyroid hormone receptor-mediated impact on vertebrate development. *Biochimica et Biophysica.* 2015, 130-141.

56. Wang, Y., Wang, Y., Dong, J., Wei Wei, W., Song, B., Min, H., et al. Developmental Hypothyroxinemia and HypothyroidismReduce Proliferation of Cerebellar Granule Neuron Precursors in Rat Offspring by Downregulation of the Sonic Hedgehog Signaling Pathway. *Mol Neurobiol.* 2014, 49:1143–1152.

57. Ahmed, O., El-Gareib, A., El-bakry, A., El-Tawab, S., & Ahmed, R. Thyroid hormones states and brain development interactions. *Int. J. Devl Neuroscience.* 2008, 26:147–209.

58. Anderson, G.W. Thyroid hormone and cerebellar development. *The Cerebellum.* 2008, 60-74.

59. Hernandez, A., Martinez, M. E., Fiering, S., Galton, V. A., & Germain, D. S. Type 3 deiodinase is critical for the maturation and function of the thyroid axis. *The J of Clinical Investigation.* 2006, 116:476-484.

60. Mullur, R., Liu, Y.-Y. and Brent, G.A. Thyroid hormone regulation of metabolism. *Physiol Rev.* 2014, 94:355-382.

61. Schmidt, M., Enthoven, L., van der Mark, M., Levine, S., de Kloet, E., & Oitzl, M. The postnatal development of the hypothalamic-pituitary-adrenal axis in the mouse. *Int Journal of Devl Neuroscience.* 2003, 21:125-132.

62. Van Middlesworth, L. Audiogenic seizures in rats after severe prenatal and perinatal iodine depletion. *Endocrinology.* 1977, 100:242.

63. Van Middlesworth, L. and Norris, C.H. Audiogenic Seizures and Cochlear Damage in Rats after Perinatal Antithyroid Treatment. *Endocrinology.* 1980, 106(6):1686-1690.

64. Simonides, W. s., Muclcahey, M. A., Redout, E., Muller, A., Zuidwijk, M. J., Visser, t. J., et al. Hypoxia-dinducible factor induces local thyroid hormone inactivation during hypoxic-ischemic disease in rats. *J Clin Invest.* 2008, 118:975-983.

65. Cunnane, S.C. and Crawford, M.A. Energetic and nutritional constraints on infant brain development: implications for brain expansion during human evolution. *Hum Evol.* 2014, 77:88–98.

66. Cunnane, S., Nugent, S., Roy, M., Courchesne-Loyer, A., Croteau, E., Tremblay, S., et al. Brain fuel metabolism, aging, and Alzheimer's disease. *Nutrition.* 2011, 27:3–20.

67. Pitts, M.W., Byrns, C.N., Ogawa-Wong, A.N., Kremer, P., & Berry, M. J. Selenoproteins in Nervous System Development and Function. *Biol Trace Elem Res.* 2014, 161:231-245.

68. Haan, J. B., Bladier, C., Griffiths, P., Kelner, M., O'Shea, R. D., Cheung, N. S., et al. Mice with a homozygous null mutation for the most abundant glutathione peroxidase, gpx1, show increased susceptibility tothe oxidative stress-inducing agents paraquat and hydrogen peroxide. *J Biol Chem* . 1998, 273:22528–22536.

69. Tsuru-Aoyagi, K., Potts, M. B., Trivedi, A., Pfankuch, T., Raber, J., Wendland, M., et al. Glutathione peroxidase activity modulates recovery in the injured immature brain. *Ann Neurol.* 2009, May; 65(5): 540–549.

70. Wirth, E., Conrad, M., Winterer, J., Wozny, C., Carlson, B., Roth, S., et al.. Neuronal selenoprotein expression is required for interneuron development and prevents seizures and neurodegeneration. *FASEB J.* 2010, Mar; 24(3):844-52.

71. Savaskan, N., Borcher, A., Brauer, A., & Kuhn, H. Role for glutathione peroxidase-4 in brain development and neuronal apoptosis: specific induction of enzyme expression in reactive astrocytes following brain injury. *Free Radic Biol Med.* 2007, 43:191-201.

72. Borcher, A., Wang, C., Ufer, C., Schiebe, H., Savaskan, N., & Kuhn, H. The role of phospholipid hydroperoxide glutathione peroxidase isoforms in murine embryogenesis. *J. Biol Chem.* 2006, 281:19655-19664.

73. Flohe, L. Selenium in mammalian spermiogensis. *Biol Chem.* 2007, 388(10):987-95.

74. Beckett, G.J. and Arthur, J.R. Selenium and endocrine systems. *Journal of Endocrinology.* 2005, 184:455-465.

75. Foresta, C., Flohe, L., Garolla, a., Ursini, F., & Maiorino, M. Male fertility is linked to the selenoprotein phospholipid hydroperoxide glutathione peroxidase. *Biol Reprod.* 2002, Sept; 67(3):967-71.

76. Brown, D.G. and Burk, R.F. Selenium retention in tissues and sperm of rats fed a torulla yeast diet. *The Journal of Nutrition.* 1973, 102:102-108.

77. Maiorino, M., Wissing, J. B., Brigelius-Flohe, R., Calabrese, F., Roveri, A., Steinert, P., et al. Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation. *J. FASEB.* 1998, 12:1359-1370.

78. Hill, K. E., Zhou, J., Austin, L. M., Motley, A. K., Ham, A.-J. L., Olson, G. E., et al. The selenium-rich C-terminal domain of mouse selenoprotein p is necessary for the supply of selenium to brain and testis but not for the maintenance of whole body selenium. *Journal of Biological Chemistry.* 2007, 282:10972-10980.

79. Soerensen1, J., Jakupoglu1, C., Beck, H., Forster, H., Schmidt, J., Schmahl, W., et al. The Role of Thioredoxin Reductases in Brain Development. *PlosOne.* 2008, 3(3): e1813.

80. Sandalova, T., Zhong, L., Lindqvist, Y., Holmgren, A., & Schneider, G. Three-dimensional structure of a mammalian thioredoxin reductase: Implications for mechanism and evolution of a selenocysteine-dependent enzyme. *PNAS.* 2001, Aug; 98(17):9533–9538.

81. Mustacich, D. and Powis, G. Thioredoxin reductase. *Biochem J.* 2000, 346:1-8.

82. Schweizer, U., Bräuer, A., Köhrle, J., Nitsch, R., & Savaskan, N. Selenium and brain function: a poorly recognized liaison. *Brain Res Rev.* 2004, Jul; 45(3):164-78. .

83. Raman, A. V., Pitts, M. W., Seyedali, A., Hashimoto, A. C., Seale, L. A., Bellinger, F. P., et al. Absence of selenoprotein P but not selenocysteine lyase results in severe neurological dysfunction. *Genes brain Behavior.* 2012, July; 11(5):61-613.

84. Hill, K. E., Zhou, J., McMahan, W. J., Motley, A. K., Atkins, J. F., Gesteland, R. F., et al. Deletion of selenoprotein P alters distribution of selenium in the mouse. *The Journal of Biological Chemistry.* 2003, Apr; 278(16):13640-13646.

85. Burk, R.F. and Hill, K.E. Selenoprotein P: An extracellular protein with unique physical characteristics and a role in selenium homeostasis. *Annu Rev Nutr.* 2005, 25:215-35.

86. Nakayama, A., Hill, K. E., Austin, L. M., Motley, A. K., & Burk, R. F. All regions of mouse brain are dependent on selenoprotein P for maintenance of selenium. *The Journal of Nutrition.* 2006, 137:690-693.

87. Hill, K. E., Zhou, J., McMahan, W. J., Motley, A. K., & Burk, R. F. Neurological dysfunction occurs in mice with targeted deletion of the selenoprotein P gene. *The Journal of Nutrition.* 2004, 134:157-161.

88. Schomburg, L., Schweizer, U., Holtmann, B., Flohe, L., Sendtner, M., & Kohrle, J. Gene disruption discloses role of selenoprotein P in selenium delivery to target tissues. *J Biochem.* 2003, 370:397-402.

89. Pitts, M., Raman, A., Hashimoto, A., Todorovic, C., Nichols, R., & Berry, M. Deletion of selenoprotein P results in impaired function of parvalabumin interneurons and alteratins in fear learning and sensorimotor gating. *Neuroscienc.* 2012, 208:58-68.

90. Peters, M., Hill, K., Burk, R., & Weber, E. Altered hippocampus synaptic function in seleoprotein P deficient mice. *Mol Neurodegener.* 2006, 1:12.

91. Burk, R. F., Hill, K. E., Olson, G. E., Weeber, E. J., Motley, A. K., Winfrey, V. P., et al. Deletion of apolipoprotein E recetor-2 in mice lowers brain selenium and causes severe neurological dysfunction and death whe a low selenium diet is fed. *J. Neuroscience.* 2007, 27:6207-6211.

92. Burk, R. F., Hill, 1. K., Motley, A. K., Winfrey, V. P., Kurokawa, S., Mitchell, S. L., et al. Selenoprotein P and apolipoprotein E receptor-2 interact at the blood-brain barrier and also within the brain to maintain an essential selenium pool that protects against neurodegeneraton. *J. FASEB.* 2014, Aug; 28(8): 3579–3588.

93. Valentine, W., Abel, Y., Hill, K., Austin, L., & Burk, R. Neurodegeneration in mice resulting from loss of functional selenoprotein P or its receptor apolipoprotein E receptor 2. *J Neuropathol Exp Neurol.* 2007, 67:68-77.

94. Weyer, C., Foley, J., Bogardus, P., Tataranni, P., & Pratley, R. Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. *J Biol Chem.* 2002, Oct 18; 277(42):39944-52.

95. Esaki, N., Takeshi, N., Tanaka, H., & Soda, K. Selenocysteine lyase, a novel enzyme that specifically acts on selenocysteine. *The Journal of Biological Chemistry.* 1982, 257(8):4386-4391.

96. Mihara, H. and Esaki, N. Selenocysteine Lyase: Mechanism, Stucture, and Biological Role. [book auth.] D.L. Hatfield, M.J. Berry and V.N. Editors Gladyshev. *Selenium Its molecular biology and Role in Human Health, 3rd* . New York Dordrecht Heidelberg London : Springer Scienc+Business Media, 2012, pp. 97-105.

97. Seale, L. A., Hashimoto, A. C., Suguru, K., Gilman, c. L., Seyedali, A., Bellinger, F. P., et al. Disruption of the selenocystein lyase-mediated selenium recyclin pathway leads to metabolic syndrome in mice. *Molecular and Cellular Biology.* 2012, 32(20):4141-4154.

98. Yeadon, J. 6 facts you should know about Nnt, C57BL/6J and diabetes. *The Jackson Laboratory.* [Online] May 28, 2014. [Cited: Jul 12, 2016.] https://www.jax.org/news-and-insights/jax-blog/2014/may/six-facts-you-should-know-about-nnt-c57bl-6j-and-diabetes.

99. Simon, M., Greenaway, S., White, J., Fuchs, H., Gailus-Durner, V., Wells, S., et al. A comparative phenotypic and genomic analysis of C57Bl/6J and C57Bl/6N mouse strains. *Genome Biol.* 2013, Jul 31;14(7):R82.

100. Freeman, H., Hugill, A., Dear, N., Ashcroft, F., & Cox, R. Deletion of Nicotinamide Nucleotide Transhydrogenase: A New Quantitive Trait Locus Accounting for Glucose Intolerance in C57BL/6J Mice. *Diabetes.* 2006, 55:2153-2156.

101. Shalini, S. and Bansal, M.P. Role of selenium in regulation of spermatogenesis: involvement of activator protein 1. *Biofactors.* 2005, 23(3):151-62.

102. Pitts, M. W., Kremer, P. M., Hashimoto, A. C., Torres, D. J., Byrns, C. N., Williams, C. S., et al. Competition between the Brain and Testes under Selenium-Compromised Conditions:

Insight into Sex Differences in Selenium Metabolism and Risk of Neurodevelopmental Disease. *Journal of Neuroscience.* 2015, 18 Nov; 35(46):15326-15338.

103. Toye, A., Lippiat, J., Proks, P., Shimomura, K., Bentley, L., Hugill, A., et al. A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. *Diabetologia.* 2005, 48:675-686.

104. Nicholson, A., Reifsnyder, P., Malcom, R., Lucas, C., MacGrgor, G., Zhang, W., et al. Diet induced obesity in two C57Bl/6 substains with intact of mutant Nicotinamde ucleotide Transhydrogenase (Nnt) gene. *Obesity.* 2010, Oct; 18(10):1902-1905.

105. Beek, L., Klinken, J., Pronk, A., Dam, A., Dirven, E., Rensen, P., et al. The limited storage capacity of gonadal adipose tissue directs the development of metabolic disorders in male C57Bl/6J mice. *Diabetologia.* 2015, 58:1601-1609.

106. Huang, T., Naeemuddin, M., Elchuri, S., Yamaguchi, M., Kozy, H., Carlson, E., et al. Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase. *Human Molecular Genetics.* 2006, 15(7):1187-1194.

107. Schomburg, L. and Schweizer, U. Hierarchical regulation of selenoprotein expression and sex-specific effects of selenium. *Biochimica et Biophysica.* 2009, 1790:1453-1462.

108. Fulop, T., Tessier, D. and Carpentier, A. The metabolic syndrome. *Pathologie Biologie.* 2006, 54:375-386.

109. Ritchie, S.A. and Connell, J.M.C. The link between abdominal obesity, metaboic syndrome and cardiovascular disease. *Nutrition, Metabolism & Cardiovascular Diseases.* 2007, 17:319-326.

110. Berry, D., Stenese, D., Zeve, D., & Graff, J. The developmental origins of adipose tissue. *Development.* 2013, 140:3939-3949.

111. Weyer, C., Foley, J., Bogardus, P., Tataranni, P., & Pratley, R. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts Type II diabetes independent of insulin resistance. *Diabetologia.* 2000, 43:1498-1506.

112. Tajaddini, M., Keikha, M., Razzazzadeh, A., & Kelishadi, R. A systematic review on the association of serum selenium and metabolic syndrome. *J Res Med Sci.* 2015, Aug; 20(8):782-789.

113. Forceville, Z., Mostert, V., Pierantoni, A., Vitous, D., Toumelin, P. L., Plouviewr, E., et al. Selenoprotein P, Rather than Glutathione Peroxidase, as a Potential Marker of Septic Shock and Related Syndromes. *European Surgical Research.* 2009, 43:338-347.

114. Olson, G. E., Whitin, J. C., Hill, K. E., Winfre, V. P., Motley, A. K., Austin, L. M., et al. Extracellular glutathione peroxidase (Gpx3) binds specifically to basement membranes of mouse renal cortex tubule cells. *Am J Physiol Renal Physiol.* 2010, 298:F1244-F1253.

115. Schmutzler, C., Mentrup, B., Schomburg, L., Hoang-Vu, C., Herzog, B., & Kohrle, J. Selenoproteins of the thyrod gland: expression, localization and possible function of glutathione peroxidase 3. *Biol Chem.* 2007, Oct: 388(10):1053.1059.

116. Howie, A. F., Walker, S. W., Akesson, B., Authur, t. J., & Beckett, G. J. Thyroidal extracellular glutathione peroxidase: a potential regulator of thyroid-hormone synthesis. *Biochem. J.* 1995, 308:713-717.

117. Baez-Duarte, B. G., Mendoza-Carrera, F., Garcia-Zapien, A., Flores-Martinez, S. E., Sanchez-Corona, J., Zamora-Ginez, I., et al. Glutathione Peroxidase 3 Serum Levels and GPX3 Gene Polymorphisms in Subjects with Metabolic Syndrome. *Archives of Medical Research.* 2014, 45:375-382.

118. Whitehead, N. S. Chapter 6 The gonad. [book auth.] NCBI Bookshelf. *Endocrinology: An Integrated Approach.* Oxford : BIOS Scientific Publishers, 2001.

119. Gillies, G.E. and McArthur, S. Estrogen action in the brain and basis for differential action in men and woman: a case for sex-specific medicines. *Pharmacological Reviews.* 2010, 62:155-198.

120. Pitts, M.W., Reeves, M.A., Hashimoto, A.C., Ogawa, A., Kremer, P., Seale, L.A., Berry, M.J. Deletion of selenoprotein M leads to obesity without cognitive deficits. *J Biol Chem.* 2013, 288:26121–26134.

121. Thomases, D. R., Cass, D. K. and Tseng, K. Y. Periadolescent exposure to the NMDA receptor antagoinis MK-801 impairs the functional maturation of local GABAergic circuits in the adult prefrontal cortex. *The Journal of Neruoscience.* 2013, 33(1):26-34.

122. Caito, S. W., Milatovic, D., Hill, K. E., Aschner, M., Burk, R. F., & Valentine, W. M. Progression of neurodegeneration and morphologic changes in the brains of juvenile mice with selenoprotein P deleted. *Brain Research.* 2011, June 29; 1398:1-12.

123. Hioki, T., Suzuki, S., Morimoto, M., Masaki, T., Tozawa, R., Morita, S., et al. Brain Testosterone Deficiency Leads to Down-Regulation of Mitochondrial Gene Expression in RAt Hippocampus Accompanied by a Decline in Peroxisome Proliferator-Activated Receptor-y Coactivator 1alpha Expression. *J Mol Neurosci.* 2014, 52:531-537.

124. Watanabe, T and Endo, A. Effects of selenium deficiency on sperm morphology and spermatocyte chromosomes in mice. *Mutat Res.* 1991, 262:93-99.

125. Schneider, M., Forster, H., Boersma, A., Wehnes, A., Sinowatz, F., Neumuller, C., et al. Mitochondrial glutathione peroxidase 4 disruption causes male infertility. *FASEB J.* 2009, 23:3233–3242.

126. Orth, M. and Bellosta, S. Cholesterol: Its Regulation and Role in Central Nervous System Disorders. *Publishing Corporation.* 2012. doi:10.1155/2012/292598 Volume 2012, Article ID 292598, 19 pages.

127. Hu, J., Zhang, Z., Shen, W.-J., & Azhar, S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis o fsteroid hormones. *Nutrition & Metabolism.* 2010, 7:47.

128. Martini, L., Celotti, F., Lechuga, M., Melcangi, R., Motta, M., Negri-Cesi, P., et al. Androgen Metablism in Different Target Tissues. *Annals New York Academy of Sciences.* 1990, 184-198.

129. Rosario, E. R., Chang, L., Beckett, T. L., Carroll, J. C., Murphy, M. P., Stanczyk, F. Z., et al. Age-related changes in serum and brain levels of androgens in male Brown Norway rats. *NeuroReport.* 2009, 20:1534-1537.

130. Pike, C. J., Nguyen, T.-V. V., Ramsden, M., Yao, M., Murphy, M. P., & Rosario, E. R. Androgen cell signaling pathways involved in neuroprotective actions. *Hormones and Behavior.* 2007, 53:693-705.

131. Jayaraman, A. and Pike, C. J. Alzheimer's Disease and Type 2 Diabetes: Multiple Mechanisma Contribute to Interactions. *Curr Diab Rep.* 2014, 14:476.

132. Cunningham, R. L., Singh, M., O'Bryant, S. E., Hall, J. R., & Barber, R. C. Oxidative stress, testosterone, and cognition among Caucasian and Mexican American men with and without Alzheimer's disease. *J Alzheimers Dis.* 2014, 40(3):563-573.

133. Yu, I.-C., Lin, H.-Y., Sparks, J. D., Yeh, S., & Chang, C. Androgen REceptor Roles in Insulin Resistance and Obesity in Males: The Linkage of Androgen-Deprivation Therapy to Metabolic Syndrome. *Diabetes.* 2014, 63:3180-3188.

134. Faingold, CL. Role of GABA abnormalities in the inferior colliculus pathophysiology – audiogenic seizures. *Hearing Research.* 2002, 168:223-237.

135. Hu, H., Gan, J. and Jonas, P. Interneurons. Fast-spiking, parvalbumin$^{+}$ GABAergic interneurons: from cellular design to microcircuit function. *Science.* 2014 , Aug; 345(6196):1255263.

136. Behrens, M., Ali, S., Dao, D., Lucero, J., Shekhtman, G., Quick, K., et al. Ketamine-induced loss of phenotype of fast-spiking interneurons is mediated by NADPH-oxidase. *Science.* 2007 , Dec 7; 318(5856):1645-7.

137. Cabungcal, J.-H., Steullet, P., Kraftsik, R., Cuenod, M., & Do, K. Q. Early-Life Insults Impair Parvalbumin Interneurons via Oxidative Stress: Reversal by N-Actylcysteine. *Biological Psychiatry.* 2013, 73:574-584.

138. Suttkus, A., Rohn, S., Jäger, C., Arendt, T., & Morawski, M. Neuroprotection against iron-induced cell death by perineuronal nets - an in vivo analysis of oxidative stress. *Am J Neurodegener Dis.* 2012, 1(2):122-9.

139. NIH, U.S. National Library of Medicine. Neurologic Diseases. *MedlinePlus.* [Online] February 1, 2017. [Cited: April 11, 2017.] https://medlineplus.gov/neurologicdiseases.html.

140. MedlinePlus. Seizures. *MedlinePlus.* [Online] [Cited: October 31, 2013.] http://www.nlm.nih.gov/medlineplus/ency/article/003200.htm.

141. Fisher, Robert S. Generalized Seizures. *Stanford School of Medicine.* [Online] 2013. http://neurology.stanford.edu/epilepsy/patientcare/videos/e_04.html.

142. Epilepsy Foundation. Partial Seizures. *Epilepsy Foundation.* [Online] 2012. http://www.epilepsyfoundation.org/aboutepilepsy/seizures/partialseizures/.

143. Fisher, Robert S. Partial Seizures. *Stanford School of Medicine.* [Online] 2013. http://neurology.stanford.edu/epilepsy/patientcare/videos/e_03.html.

144. Gelastic&Dacrystic. *Epilepsy Foundation* . [Online] 2012. http://www.epilepsyfoundation.org/aboutepilepsy/seizures/Gelastic-and-Dacrystic-Seizures.cfm.

145. Non-epileptic Seizures. *Epilepsy Society* . [Online] 2013. http://www.epilepsysociety.org.uk/non-epileptic-seizures#.UqPuy0rueSo.

146. Epilepsy Fact Sheet . *World Health Organization.* [Online] February 2016. [Cited: July 29, 2016.] http://www.who.int/mediacentre/factsheets/fs999/en/.

147. Dogini, D.B., Avansini, S.H., Vieira, A.S. and Lopes-Cendes, I., MicroRNA regulation and dysregulation in epilepsy. *Frontiers in Cellular Neuroscience*. 04 Oct 2013,Vol 7 Article 172:1-8.

148. Treatment Options: Medication. *Epilepsy Foundation.* [Online] 2013. http://www.epilepsyfoundation.org/aboutepilepsy/treatment/medications/index.cfm.

149. Brain Stimulation Therapies. *National Institute of Mental Health.* [Online] 2013. http://www.nimh.nih.gov/health/topics/brain-stimulation-therapies/brain-stimulation-therapies.shtml.

150. Crepin, S., Godet, B., Chassain, B., Preuz, P.-M., & Desport, J.C. Malnutrition and epilepsy: A two-way relationship. *Clinical Nutrition*. 2009, 28(3):219-225.

151. Ketonic Diet. *Epilepsy Foundation.* [Online] 2013. http://www.epilepsyfoundation.org/aboutepilepsy/treatment/ketogenicdiet/.

152. Alternative and Complementary Therapies. *Canadian Epilepsy Alliance.* [Online] 2008. http://www.epilepsymatters.com/english/trealternative.html.

153. Waterston, R.H. and et al. Initial sequencing and comparative analysis of the mouse genome. *Nature.* 2002, 5 Dec, 420:520-562.

154. Loscher, W. Critical review of current animal models of seizures and epilepsy used in the discovery and development of new antiepileptic drugs. *Seizure.* 2011, 20:359-368.

155. Auvin, S., Pineda, E., Shin, D., Gressens, P., & Mazarati, A. Novel Animal Models of Pediatric Epilepsy. *Neurotherapeutics.* 2012, 9:245-261.

156. Grone, B. and Baraban, S. Animal Models of Epilepsy: Legacies and New Directions. *Nature Neuroscience.* 2015, 18:339-343.

157. Marx, V. Models: stretching the skills of cell lines and mice. *Nature Methods.* 2014, Jun.; 11(6):617-620.

158. Jucker, M. The benefits and limitations of animal models for translational research in neuordegenerative diseases. *Mature Medicine.* 2010, Nov; 16(11):1210-1214.

159. Perel, P., Robers, I., Sena, E., Wheble, P., Briscoe, C., Sandercock, P., et al. Comparison of treatment effects between animal experments and clinical trials; systematic review. *MBJ.* 2006, 334:197.

160. Naziroğlu, M., Kutluhan, S. and Yilmaz, M. Selenium and topiramate modulates brain microsomal oxidative stress values, Ca2+-ATPase activity, and EEG records in pentylentetrazol-induced seizures in rats. *J Membr Biol.* 2008, Sept.-Oct.; 225(1-3):39-49.

161. Shin, E., Jeong, J., Chung, Y., Kim, W., Ko, K., JH, B., et al. Role of oxidative stress in epileptic seizure. *Neurochemistry International.* 2011, 50:122-137.

162. Oztas, B., Akgul, S. and Seker, F.B. Gender difference in the influence of antioxidants on the blood-brain barrier permeability during pentylenetetrazol-induced seizures in hyperthermic rat pups. *Biol Trace Elem Res.* 2007, Jul.;118(1):77-83.

163. Rehni, A., & Singh, T. Selenium induced anticonvulsant effect: a potential role of prostaglandin E(1) receptor activation linked mechanism. *J Trace Elem Med Biol.* 2013, Jan.; 27(1):31-9.

164. Jones, N., Nguyen, T., Corcoran, N., Velakoulis, D., Chen, T., Grundy, R., et al.Targeting hyperphosphorylated tau with sodium selenate suppresses seizures in rodent models. *Neurobiol Dis.* 2012, Mar.; 45(3):897-901.

165. Savaskan, N., Bräuer, A., Kühbacher, M., Eyüpoglu, I., Kyriakopoulos, A., Ninnemann, O., et al. Selenium deficiency increases susceptibility to glutamate-induced excitotoxicity. *FASEB J.* 2003, Jan.;17(1):112-4.

166. Yürekli, VA and Nazıroğlu, M. Selenium and topiramate attenuates blood oxidative toxicity in patients with epilepsy: a clinical pilot study. *Biol Trace Elem Res.* 2013, Vols. May;152(2):180-6.

167. Ashrafi, M., Shabanian, R., Abbaskhanian, A., Nasirian, A., Ghofrani, M., Mohammadi, M., et al. Selenium and intractable epilepsy: is there any correlation? *Pediatr Neurol.* 2007, Jan.; 36(1):25-9.

168. Ashrafi, M., Shams, S., Nouri, M., Mohseni, M., Shabanian, R., Yekaninejad, M., et al. A probable causative factor for an old problem: selenium and glutathione peroxidase appear to play important roles in epilepsy pathogenesis. *Epilepsia.* 2007, Sept.; 48(9):1750-5.

169. Seven, M., Basaran, S., Cengiz, M., Unal, S., & Yuksel, A. Deficiency of selenium and zinc as a causative factor for idiopathic intractable epilepsy. *Epilepsy Res.* 2013, Mar.;104(1-2):35-9.

170. Olson, G. E., Winfrey, V. P., NagDas, S. K., Hill, K. E., & Burk, R. F. Selenoprotein P is required for mouse sperm development. *Biology of Reproduction. 2005,* 73:201-211.