

**THE ROLE OF SELENOPROTEINS IN PROTECTION AGAINST
METHAMPHETAMINE-INDUCED DOPAMINERGIC NEURODEGENERATION**

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ABSTRACT

Methamphetamine (MA) increases oxidative stress through actions on dopaminergic transmission. Selenium (Se) is an antioxidant trace element that is necessary for normal brain functions. Previous studies have shown that Se protects against MA neurotoxicity and is necessary for proper dopamine function. The specific aim of the project is to determine the role of antioxidant selenoproteins, including the glutathione peroxidases (GPXs), in protecting dopaminergic neurons from MA toxicity *in vitro* and *in vivo*.

In SH-SY5Y cells, MA decreased protein levels for GPX1 and GPX4. However, both proteins were upregulated with increasing Se concentration. GPX enzymatic activity was increased by Se concentrations and decreased by MA and correlated with GPX protein levels. MA reduced total intracellular GSH levels at lower Se concentrations, while the oxidized fraction of GSH was increased at higher Se levels. Extracellular GSH was increased in 0 nM Se only, with no changes from MA or any changes in the oxidized fraction of GSH. Additionally, MA increased ROS levels in cell cultures grown in 0 nM Se, but Se supplementation prevented the increase. Our *in vivo* results show that GPX1 and GPX4 increase with Se, whereas, DAT and TH expression do not appear to change.

The results of our study indicate that a MA-induced reduction in GPX levels can contribute to increased oxidative stress. GPX reduction can be prevented increasing Se levels. These findings have important implications for treating patients with acute methamphetamine toxicity.

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LIST OF ABBREVIATIONS

5HT	serotonin
BBB	blood-brain barrier
CNS	central nervous system
DA	dopamine
DAT	Dopamine transporter protein
DOPAC	3,4-Dihydroxyphenylacetic acid
GSH	reduced glutathione
GSSG	oxidized glutathione
GPX	glutathione peroxidase
MA	methamphetamine
MAOB	monoamine oxidase B
NAcc	nucleus accumbens
RNS	reactive nitrogen species
ROS	reactive oxygen species
Se	selenium
Sec	selenocysteine
SN	substantia nigra
tGSH	total glutathione
TH	tyrosine hydroxylase
VMAT2	vesicular monoamine transporter 2
VSTR	ventral striatum
VTA	ventral tegmental ar

CHAPTER 1

INTRODUCTION

Methamphetamine (MA) is an illicit psychostimulant drug that has become an international public health problem. MA is the second most widely abused drug after cannabis, and the number of MA users exceeds both heroin and cocaine [1]. The surge of MA abuse can be attributed to several factors, including its long lasting euphoric effect, relative ease of synthesizing the drug from inexpensive over-the-counter ingredients, and widespread distribution of products from “mom and pop” laboratories and “super lab” organizations [2]. The MA epidemic is a primary concern for Hawaii’s communities and healthcare. Minority populations are at a higher risk, particularly racial/ethnic groups such as Native Hawaiians and Pacific Island Peoples [3]. In regards to healthcare, an estimated emergency department visits for MA abuse increased by almost 50% between 2004 and 2005 [3]. Understanding the mechanisms of MA toxicity and finding viable treatments are both important to ensuring both individual recovery as well as decreasing the financial and social consequences of the MA epidemic.

MA is a potent neurotoxin of the amphetamine class of drugs, which are known to have stimulant, euphoric, and in some cases hallucinogenic properties [4]. MA’s additional methyl group increases its lipophilic nature compared to its parent compound amphetamine, allowing the molecule to easily cross the blood-brain barrier (BBB) into the central nervous system (CNS) [5]. Like amphetamines, MA stimulates the release of monoamine neurotransmitters and interferes with the neurotransmitter reuptake mechanism [6]. The half-life of MA is approximately 10 h compared to the 90-minute half life of cocaine [5] and acute effects can last up to 8 h for a single moderate dose [4].

Immediately after taking the drug, users experience a euphoric sensation or “high,” increased energy, hypersexuality, decreased anxiety, and elevated interest in environmental stimuli [5, 7]. These pleasurable sensations can lead to habitual use or abuse. In addition, MA stimulates the sympathetic nervous system, which can cause tachycardia, peripheral hyperthermia, and tachypnea [5]. Withdrawal from MA can lead to psychotic behaviors, such as paranoia, aggression, and an intense craving for the drug [6]. Furthermore, chronic use has been shown to cause long-lasting brain damage. Clinical studies found a significant reduction in neuronal density and neuronal content within the brain of abstinent MA users [8].

MA affects monoamine neurotransmitter systems, such as dopamine (DA), norepinephrine, epinephrine, and serotonin (5HT). This study will primarily focus on how MA impacts the dopaminergic pathway. Dopaminergic neurons are found within the ventral tegmental area (VTA) and the substantia nigra (SN) of the midbrain. VTA dopaminergic neurons innervate the ventral striatum (VSTR), cortical areas, and the limbic system and regulate emotional behavior, motivation, and reward. SN dopaminergic neurons project to the dorsal lateral striatum

and control motor function [9]. These midbrain dopaminergic neurons are of great clinical interest because of their involvement in mental disorders and Parkinson's disease.

MA induces dopaminergic neurodegeneration by eliciting its effects through DA. Two models have been suggested to explain the role of DA: the exchange diffusion model and the weak base model. The exchange diffusion model proposes that MA disrupts the reuptake mechanism of DA by reversing the conformation of the dopamine transporter (DAT), thereby causing DAT to release excess DA into the synaptic cleft [10]. The prolonged presence of extracellular DA results in its auto-oxidation producing reactive oxygen species (ROS) and reactive nitrogen species (RNS). However, this model does not explain how only dopaminergic neurons are damaged and not neighboring non-dopaminergic neurons. The weak base model predicts that MA enters terminals through DAT and disrupts the proton gradient necessary for function of vesicular monoamine transporter 2 (VMAT2) [11]. Surplus DA into the cytoplasm auto-oxidizes and causes intraneuronal oxidative damage. The weak base model suggests that extracellular DA is not necessary to elicit neurodegeneration of dopaminergic neurons. Overall, MA neurotoxicity involves oxidative stress possibly caused by DA auto-oxidation and promoting neurotoxic ROS [10].

DA release and subsequent enzymatic oxidation, DA auto-oxidation, and increased mitochondrial function are the general mechanism by which MA produces reactive species. DA is metabolized by monoamine oxidase B (MAOB) to 3,4-Dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide (H_2O_2) [12]. Although these molecules are not reactive, H_2O_2 can further react with transition metal ions to produce hydroxyl radicals ($\bullet OH$). DA can also undergo non-enzymatic oxidation by O_2 to form semi-ubiquinones and superoxide anion (O_2^-) [13]. These highly reactive hydroxyl radicals can damage amino acids, phospholipids, and nucleic acids. MA can also increase extracellular glutamate concentrations, which also increases production of reactive species [13]. Lastly, studies have shown that MA disrupts mitochondrial function by altering complex II within the electron transport chain [14]. Ultimately, the increase production of reactive species leads to oxidative stress that can cause neuronal damage and trigger neuronal apoptosis.

Neurophysiological changes caused by MA toxicity in the brain of human MA users are similar to those observed in experimental animals. Post-mortem studies reveal that tyrosine hydroxylase (TH), DAT, and DA levels are reduced in caudate-putamen and in the nucleus accumbens (NAcc) of chronic MA users [7, 15]. TH and DAT are common markers used to investigate terminal integrity. Likewise in mice, repeated exposure to MA exhibited a decreased in TH [16], TH activity, and DAT protein levels [16, 17] in striatum.

Selenium (Se) is an essential micronutrient that improves clinical responses to neurodegenerative diseases associated with increase oxidative stress [18]. In regards to MA-induced neurodegeneration, animal studies have demonstrated that Se supplementation prevents the reduction of DA and its metabolites in caudate nucleus of mice caused by MA [19]. In addition, Se supplementation has been shown to attenuate the formation of 3-nitrotyrosine (3-NT),

biomarker for RNS, in mouse striatum [20]. Furthermore, animals on a Se-deficient diet experience a significant increase in DA turnover in hippocampus [18] and prefrontal cortex [21].

Although studies demonstrate the protective effects of Se against MA toxicity, little is known about the mechanism of Se protection. The function of Se is carried out by selenoproteins. Se is incorporated into selenoproteins as selenocystine (Sec), the 21st amino acid [22]. Many of the members of the selenoprotein family are involved in redox reactions and have antioxidant properties. The glutathione peroxidases (GPX) are known antioxidant enzymes that contain one Sec at the active site [23]. GPXs utilize the substrate glutathione (GSH) to reduce hydrogen peroxides and lipid hydroperoxides. Research has yet to identify which selenoproteins are involved in protection against MA, however, some studies have suggested GPX activity as a contributor.

In this study, we investigate how Se supplementation affects selenoprotein synthesis within dopaminergic neurons. In addition, we will identify which selenoproteins are involved in protection against MA-induced toxicity and elucidate their role in preventing neurodegeneration *in vitro* and *in vivo*. In order to manipulate Se levels in culture media, we used a recently published formula that is similar to Gibco B27 supplement [24]. The serum-free supplement media allows the alteration of Se while maintaining neuronal cells in culture. For the *in vivo* experiments, we raised mice on different Se diets and did a MA treatment. Our study shows that several selenoproteins increase with Se supplementation, and that GPX 1 and GPX4 are involved in protecting against MA-induced oxidative stress.

CHAPTER II

MATERIALS AND METHODS

Serum-free media

We prepared serum-free media using a media supplement recently described by Schweizer and colleagues [24]. This supplement is similar to the B27 supplement [25] available commercially (Gibco), and is composed of the same reagents. These include: biotin, L-carnitine, ethanolamine, D (+)-galactose, putrescine dihydrochloride, albumin (bovine), catalase, glutathione, reduced superoxide dismutase, apo-transferrin, Na₂SeO₃, ZnSO₄, CuSO₄, MnCl₂, NH₄VO₃, corticosterone, linoleic acid, linolenic acid, progesterone, retinyl acetate D, L- α -tocopherol D, L- α -tocopherol acetate, Lipoic acid, Insulin (human), and 3,3', 5-triiodo-L-thyronine (T3). All supplemental reagents were purchased from Sigma, and were added directly to Neurobasal media (Invitrogen). The sodium selenite (Na₂SeO₃) was added to the media last at 0, 10, or 100 nM concentrations for Se deplete, Se low, and Se supplemented conditions.

Cell culture conditions

SH-SY5Y cells were plated in DMEM (Gibco) with 10% FBS (Gibco). After 24 h, the media was changed to Neurobasal Medium (Gibco) containing B27 supplement (Gibco), with the addition of glutamine (2mM), penicillin (1000U/ml), and streptomycin (100 μ g/ml). Cells used for were grown in this media for two days, and retinoic acid in the B27 supplement formulation induced neuronal differentiation. Cells were then subsequently exposed to media consisting of Neurobasal with the Schweizer supplement with sodium selenite adjusted to 0 nM, 10 nM, and 100 nM Se for one week before harvesting protein. Human cerebral spinal fluid has been reported to contain between 3.4-30 nM Se [26] with the 12.5 nM Se as the median. For our *in vitro* experiments, we considered 10 nM Se without MA as our control, which is within the physiological range.

Cells were differentiated in Neurobasal with Schweizer supplement containing varying concentrations of Se for two days for the dose response curve and two weeks for all other experiments. Longer differentiation periods for SH-SY5Y cells increase expression of TH and result in a phenotype more closely resembling dopaminergic neurons [27]. Following differentiation, media was changed to that containing methamphetamine hydrochloride (100 μ M) 24 h prior to harvesting cells for RNA or protein.

Methamphetamine treatment

After Se treatment, cell were exposed to (+)-Methamphetamine hydrochloride solution (Sigma) containing 0, 10 or 100 μ M MA for 24 h before harvesting for proteins.

RNA isolation and qPCR analysis

RNA was harvested with TRIZOL (Invitrogen) reagent and purified with RNeasy Mini Kit (Qiagen). cDNA was synthesized using Applied Biosystem (Foster City, CA) High Capacity cDNA Reverse Transcription Kit. qPCR reactions comprised of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), oligonucleotide primers (IDT) and cDNA. Experiments were conducted on a Roche LightCycler 480 II.

Western blot analysis

Protein was harvested with CellLytic solution and run on a 10-20% gradient Criterion Tris-HCL gels (Bio-Rad). Protein were identified with anti-rabbit BiP (Cell Signaling), anti-rabbit SelK (Sigma), SelM (Sigma), SelS (Sigma), GPX4 (AbFrontier), anti-goat GPX1 (GenWay), and anti-alpha tubulin (Novus) for *in vitro* studies. Proteins were identified with TH (Cell Signaling), DAT (Millipore), GPX4 (Epitomics) and anti-goat GPX1 (GenWay) for *in vivo* studies. Protein was imaged using the Odyssey Infrared Imaging System (LI-CORE, Lincoln, NE). Separate western blots had samples from each condition, and samples on replicate blots were normalized to the 10 nM Se, 0 μ M MA sample within the same blot.

Determination of GPX activity by GPX-340 assay

SH-SY5Y cells were incubated with 0, 10, or 100 nM Se for one week followed by a 24-h MA treatment. Protein concentration was determined by Bradford assay. GPX activity was determined using BIOXYTECH GPX-340 assay kit (Oxis) per manufacturer's instructions. Protein was harvested with CellLytic solution and mixed with 25 μ L assay buffer, 25 μ L NADPH, and 25 μ L 1:10,000 tert-Butyl Hydroperoxide. The decrease in absorption at 340 nm was monitored every 2 sec for 5 min. The rate of decrease is directly proportional to GPX activity. Results are displayed as GPX activity /protein concentration.

Determination of GSH and GSSG by GSH/GSSG-412 assay

Glutathione (GSH) and oxidized glutathione (GSSG) concentration were determined using Oxis GSH/GSSG-412. SH-SY5Y cells were treated with 0, 10, or 100 nM Se for one week followed by a 24-h MA treatment. Protein was harvested with proteinase inhibitor cocktail set III (Calbiochem) diluted in 1X PBS and dissociated by sonication. An aliquot of protein extract was mixed with 1-MAyl-vinul-pyridium trifluoromane sulfonate (M2VP) in HCl (Oxis) to be used in determining GSSG concentration. Protein extract and protein aliquot containing M2VP were mixed with 20 μ L of 1X Glutathione reductase (Sigma), 20 μ L Chromagen (Oxis), 17 μ L assay buffer and 20 μ L of 0.25 mM NADPH (Oxis). The increase in absorbance at 412 nm wavelength was monitored every 12 sec for 5 min at 412 nm wavelength. Results were transformed to total GSH (tGSH) and GSSG concentrations, calculated by comparing to the standard curve provided

by the kit. Results are displayed as GSH concentration / protein concentration. Protein concentration was determined by ND-1000 Nanodrop spectrophotometer (Thermo Scientific).

Determination of oxidative stress

Oxidative stress was measured using a 2', 7'-dichlorofluorescein diacetate (DCFH-DA) fluorescent assay. SH-SY5Y cells were treated with 0, 10, or 100 nM Se for one week followed by a 24-h MA treatment. Protein extract was added to 100 μ L of 5 μ M DCFH-DA dissolved in serum-free media supplement. The increase in fluorescence was monitored every 2 min for 1 h at 485 nm excitation and 535 nm emission. Results were transformed to amount (nmol) of product/min/mg protein using a standard curve generated with the following concentrations of DCF dissolved in Neurobasal with Schweizer supplement: 10 nM, 50 nM, 100 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M.

Animals

Animals were provided food and water as needed per University of Hawaii veterinary protocol. The University of Hawaii Institutional Animal Care and Use Committee approved all animal protocols. Adult male and female C57BL/6 mice weighing 20-35 g were used in this study. Animals were divided into six groups and their diet and treatment are described in Table 1. At three weeks of age, animals were maintained on diets containing low (0.0 ppm), moderate (0.25 ppm) or high (1 ppm) Se for 21 weeks.

Group	Diet	MA	N	Male	Female
I	Deficient Se (0.0 ppm)	-	12	5	6
II		+	12	6	6
III	Moderate Se (0.25 ppm)	-	12	6	6
IV		+	12	6	6
V	High Se	-	12	6	6
VI		+	12	6	6

Table 1. Summary of dietary Se

Methamphetamine treatment

At 23 weeks of age group II, VI, and VI received daily intraperitoneal (IP) injections of 1 mM (+)-Methamphetamine hydrochloride (Sigma) in saline (5mg/kg body weight) for five days, one injection per day. As a control, group I, III, and V received IP injections of saline. Body weight was measured during MA/saline treatment. Animals were anesthetized using tribromoethanol and decapitated 24 h after last injection.

Tissue harvest

Brains were bisected midline upon tissue harvest and flash-frozen in liquid nitrogen. Midbrain and VSTR was dissected from the right hemisphere and pulverized into a powder consistency for western blot analysis.

Statistical analysis

Experimental groups are expressed as means \pm SEM. Differences between samples with one variable were analyzed using 1-way ANOVA with Bonferoni's posthoc test, and differences between group means in experiments with more than one variable were compared using 2-way ANOVA with Bonferoni's posthoc test. P values < 0.05 are considered statistically significant.

CHAPTER III

RESULTS

Selenoprotein expression in response to Se supplementation

We first examined which selenoproteins changed with different Se levels. We hypothesized that Se could be protective from MA by increasing expression of none or more selenoproteins. Therefore, we first examined which selenoproteins increased with increasing media Se levels. We observed that most selenoproteins exhibit a significant increase in RNA synthesis when supplemented with 10nM Se compared to 0 nM Se (Fig. 1A). Only selenoprotein W (Sel W) and selenoprotein S (Sel S) increased in RNA expression with 100 nM compared to 10 nM Se (Fig.1B). We also found that protein concentrations increased in proportion to Se supplementation for all selenoproteins tested (Fig. 2). These included the glutathione peroxidases GPX1 and GPX4. We reasoned that these selenoenzymes could protect against MA-induced free radical generation, and thus further examined their responses to MA.

GPX1 and GPX4 proteins in response to MA

We detected no change in GPX1 protein expression with increasing MA concentration in undifferentiated cells (Fig 3A). However, we did see a decrease in protein expression with increasing MA in differentiated cells with a significant decrease at 100 μ M MA (Fig. 3B). We observed a similar pattern with GPX4 where protein expression is unchanged in undifferentiated cells (Fig. 3C) whereas GPX4 decreased in differentiated cells with a significant decrease at 100 μ M MA (Fig. 3D).

We challenged Se supplemented SH-SY5Y cells with MA and measured protein expression. In Figure 4A, we observed a small increase from 0 to 10 nM Se and a large increase from 10 to 100 nM Se. In 0 nM Se, MA significantly decreased GPX1 expression compared to untreated controls. There was an overall significant effect of Se on GPX1 levels. We also measured GPX4 protein concentration and saw a similar pattern to GPX1 (Fig. 4B) where there was a slight increase from 0 to 10 nM Se and a greater increase from 10 to 100 nM Se. Se had a significant affect overall. In addition, GPX4 expression significantly decreased in 0 nM Se with MA compared to untreated conditions.

GPX activity

We next investigated if increased GPX protein expression resulted in an overall increase in GPX activity. We found that activity followed a similar pattern to protein expression, where GPX activity increased with Se supplementation (Fig. 5). However, 100 μ M MA decreased GPX activity.

Glutathione levels

We next examined if GPX activity changes resulted in differences in glutathione (GSH). We measured intracellular and extracellular GSH content. GSH is a co-factor for GPX enzyme and increase in GSH content suggests the increase utilization of GSH by GPX enzymes to reduce oxidative stress. Figure 6A shows that total GSH (tGSH) content was higher in 0 nM and 10 nM Se media and decreased with Se supplementation. The intracellular oxidized GSH fraction ([GSSG]:[tGSH]) decreased in 10 nM Se media compared to 0 nM Se. In 10 nM and 100 nM Se media, the addition of MA significantly increased the [GSSG]/[tGSH] ratio (Fig. 6B).

Because glutathione is a component of the extracellular media [24], we also collected the cell culture media at the time of cell harvest in order to measure GSH and GSSG media levels. Figure 6C demonstrates a significant increase in 0 nM Se media compared to 10 nM Se media in untreated conditions. In 10 nM and 100 nM Se media, tGSH content was close to the concentration of GSH added to media (3.6 μ M represented by horizontal dashed line in Fig. 6C). This indicates that GSH is being released extracellular from cells grown in Se-depleted conditions. In 10 nM Se media, the tGSH was significantly lower when MA was added. As for the extracellular oxidized GSH fraction, there was no alteration by Se or MA (Fig. 6D).

ROS generation

We tested if decreased GPX activity levels resulted in greater ROS generation. Figure 7 shows ROS generation in response to Se and MA. In 0 nM Se we observed a significant increase in the presence of MA. However, there was no increase in ROS in cell treated with MA in 10 nM and 100 nM Se media.

Expression of GPX1 and GPX4 protein in midbrain and ventral striatum after MA treatment

To determine the physiological relevance of our *in vitro* results, we measured GPX1 and GPX4 levels in dopaminergic neurons found within the mesolimbic pathway. We observed an increase in GPX1 protein expression with Se supplementation in midbrain (Fig.8A). In addition, there was an overall significant effect of Se between low and high Se conditions in VSTR (Fig. 8B) of C57BL/6 mice raised on diets containing low, moderate, or high Se. The increase in protein expression with supplementation reflects those observed in SH-SY5Y cells. However, in contrast to the *in vitro* studies, we did not observe a reduction in response to MA in either brain region. As for GPX4, we observe no change midbrain and VSTR. (Fig.9A and 9B).

Expression of DAT and TH protein in midbrain and ventral striatum after MA treatment

We then measured DA makers DAT and TH to determine if Se and MA altered the physiology of the mesolimbic pathway. We detected no changes in DAT levels with Se supplementation in the presence of MA in midbrain (Fig. 10A) and VSTR (Fig. 10B). In addition we observed no changes in TH expression in midbrain (Fig. 11A) and VSTR (Fig. 11B).

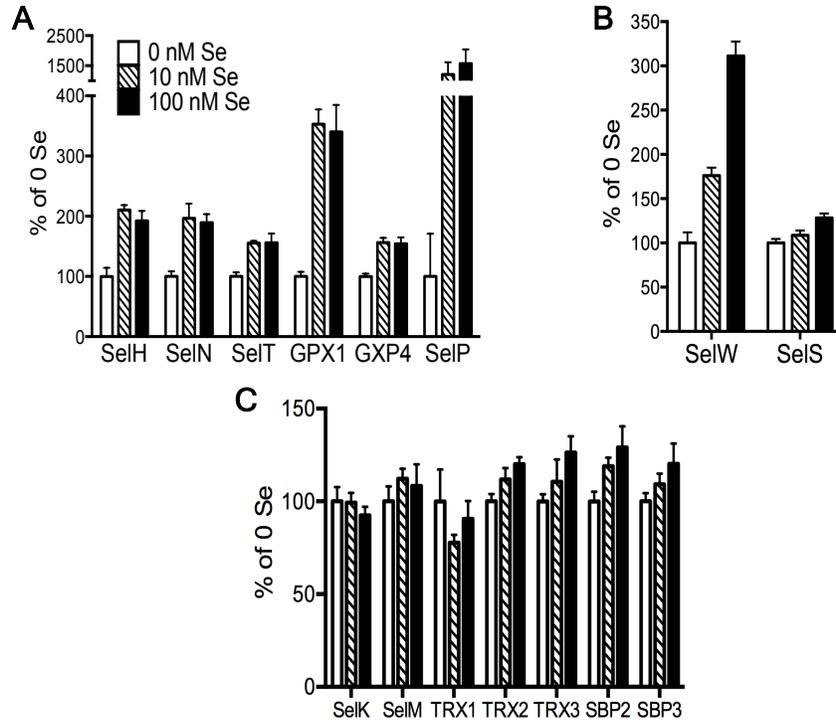


Figure 1. RNA expression in response to variations in Se concentration. (A) Increased from 0 to 10 nM Se. (B) Increase from 10 to 100 nM Se. (C) No change.

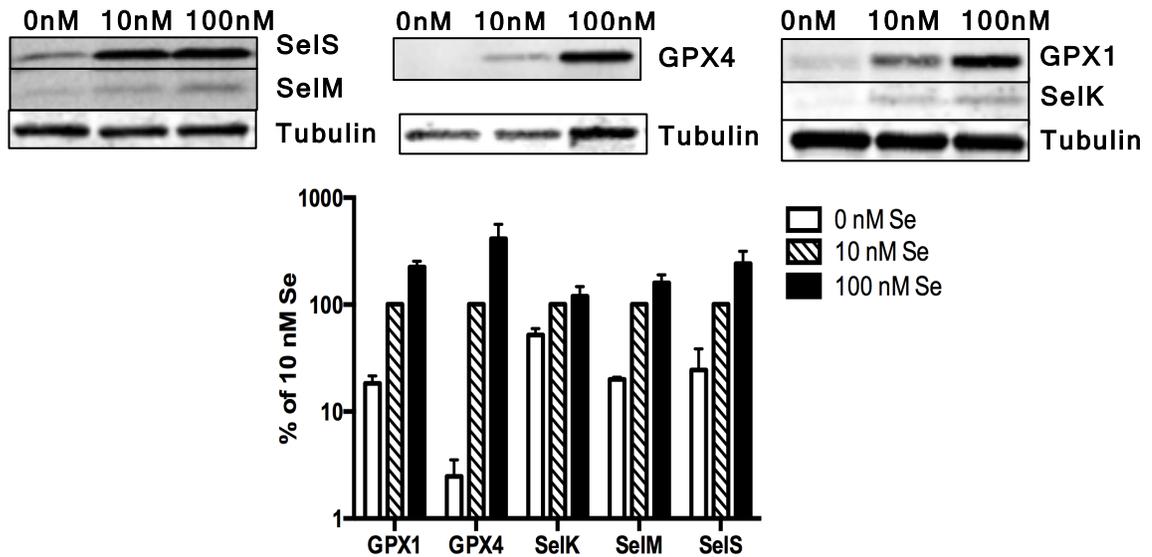


Figure 2. Protein expression increased with Se supplementation for all selenoproteins. Above: Representative western blot of GPX1, GPX4, SelK, SelM, SelS from SH-SY5Y cells grown in either 0, 10, 100 nM Se.

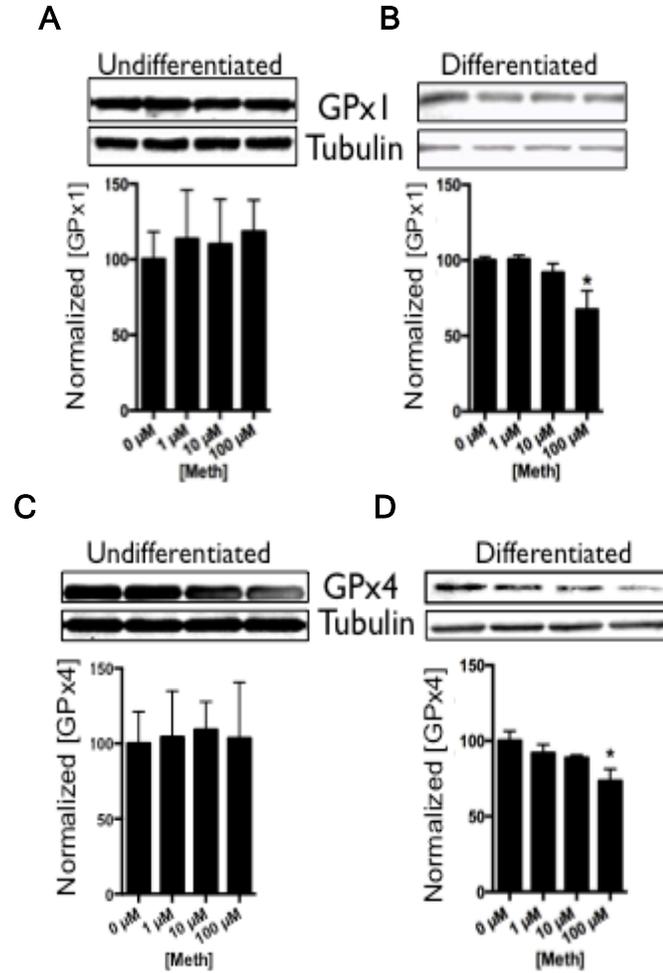


Figure 3. MA decreases GPX protein in a concentration-dependent manner.

(A) GPX 1 protein expressions in undifferentiated cells

(B) GPX1 protein expression in differentiated cells

(C) GPX4 protein expression in undifferentiated cells

(D) GPX4 protein expression in differentiated cells

* Indicates $p < 0.05$ (One-Way ANOVA with Bonferoni's posthoc test).

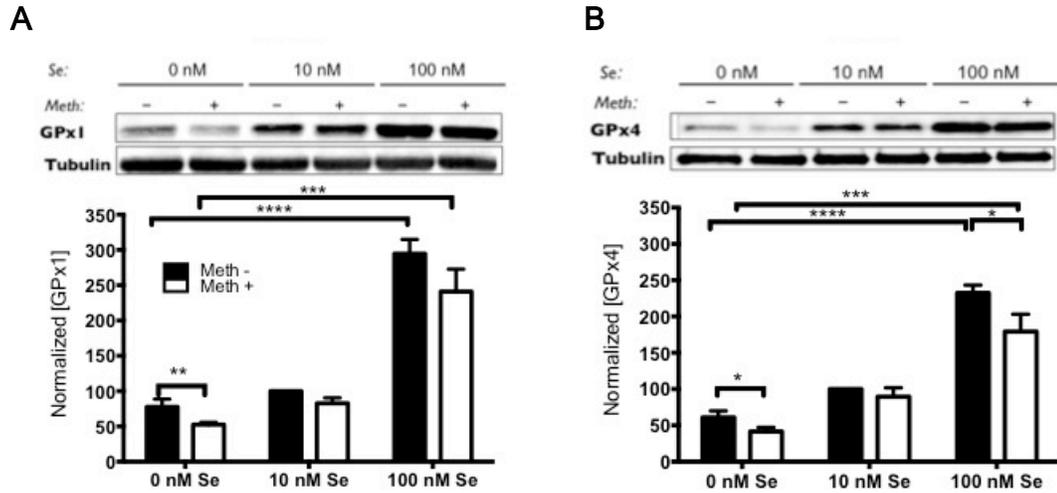


Figure 4. MA and Se alter GPX1 and GPX4 protein levels in vitro. Above: Representative western blot of GPX1 (A) and GPX4 (B) from SH-SY5Y cells grown in 0, 10, or 100 nM Se, with or without 100 μ M MA. Bars show mean \pm SEM for four replicate cultures per condition. Below: Graph of GPX1 protein mean \pm SEM measured by optical density of western blot bands. * indicates $p < 0.05$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$ (Two-Way ANOVA with Bonferoni's posthoc test)

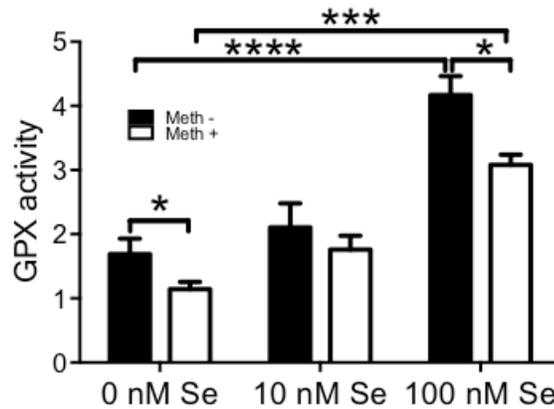


Figure 5. MA decreases GPX activity. **** Indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.001$, and * indicates $p < 0.01$ (Two-Way ANOVA with Bonferoni's posthoc test)

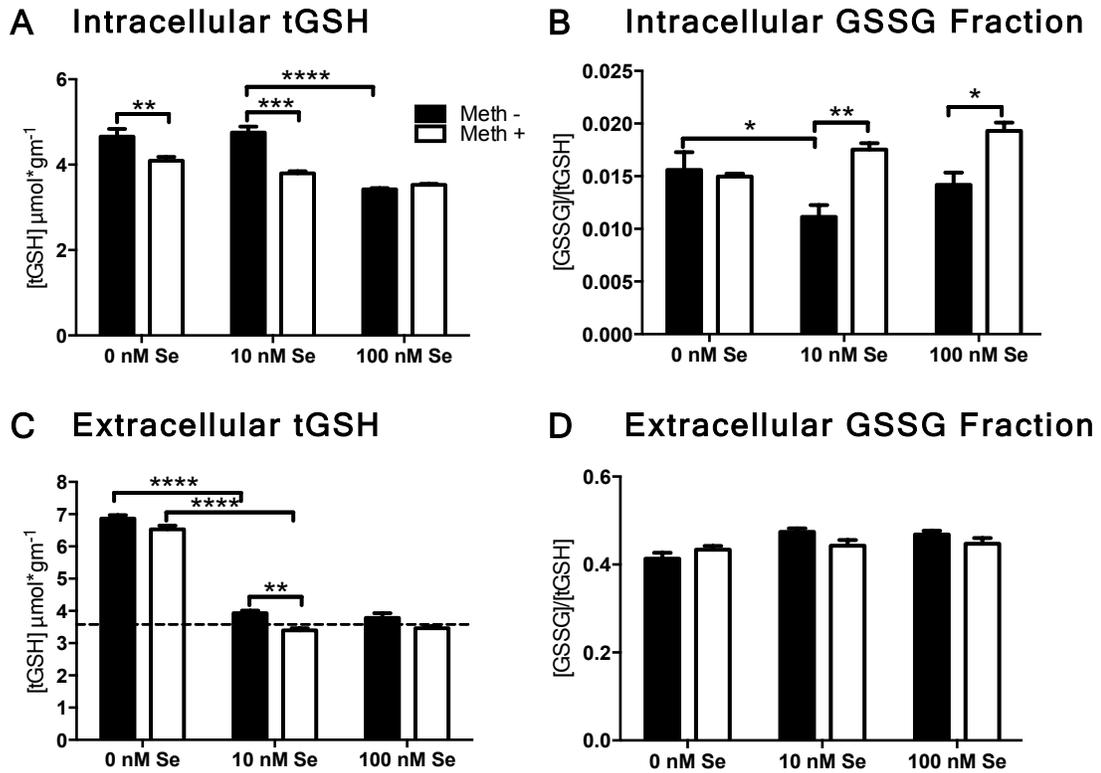


Figure 6. MA- and Se-induced changes in total GSH (tGSH) content and oxidized GSH (GSSG) fraction. MA- and Se-induced changes in total GSH (tGSH) content and the ratio of oxidized GSH (GSSG) to tGSH. (A, B) Intracellular tGSH and the ratio of intracellular GSSG:tGSH. (C, D) Extracellular tGSH and the ratio of extracellular GSSG:tGSH. Bars show mean \pm SEM for four replicate cultures per condition. **** Indicates $p < 0.0001$, *** indicates $p < 0.001$, ** indicates $p < 0.001$, and * indicates $p < 0.01$ (Two-Way ANOVA with Bonferoni's posthoc test)

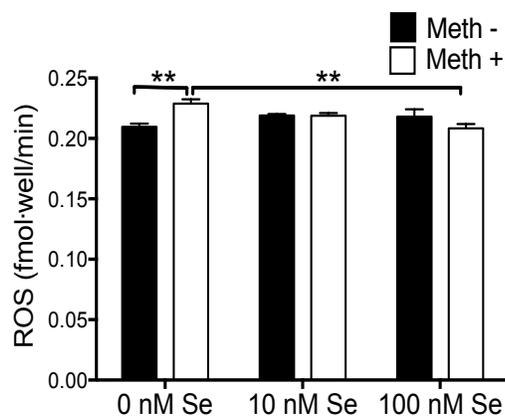


Figure 7. MA-induced generation of ROS in SH-SY5Y cells in 0 nM Se. Bars show mean \pm SEM for four replicate cultures per condition. ** Indicates $p < 0.01$ (Two-Way ANOVA with Bonferoni's posthoc test)

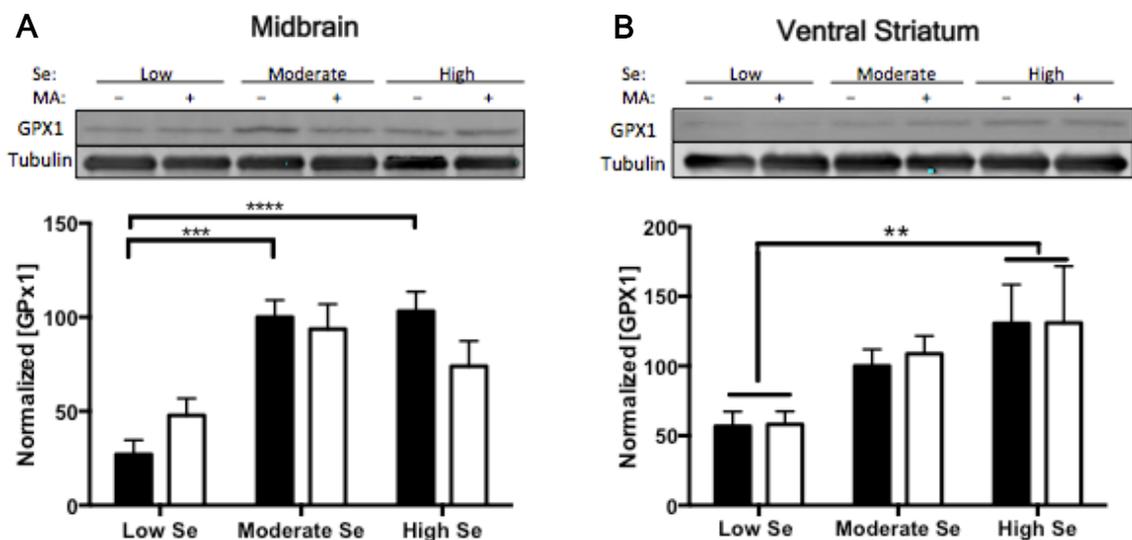


Figure 8. Effects of MA (5 x 5 kg/mg, i.p.) on GPX1 concentration in midbrain (A) and VSTR (B) of adult male and female C57BL/6 mice. Above: Representative western blot of GPX1 from animals raised on Se diet for 21 weeks before MA treatment. Bars show mean ± SEM for 5-6 animals/group. Graph of GPX1 protein mean ± SEM measured by optical density of western blot bands. ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates $p < 0.0001$ (Two-Way ANOVA with Bonferoni's posthoc test)

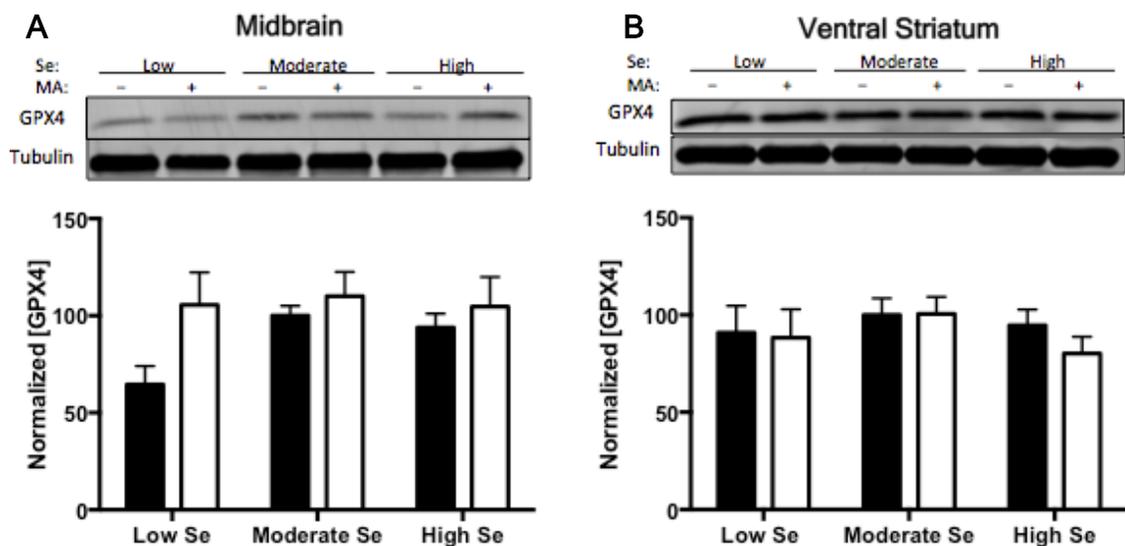


Figure 9. Effects of MA (5 x 5 kg/mg, i.p.) on GPX4 concentration in midbrain (A) and VSTR (B) of adult male and female C57BL/6 mice. Above: Representative western blot of GPX4 from animals raised on Se diet for 21 weeks before MA treatment. Bars show mean ± SEM for 5-6 animals/group. Graph of GPX4 protein mean ± SEM measured by optical density of western blot bands.

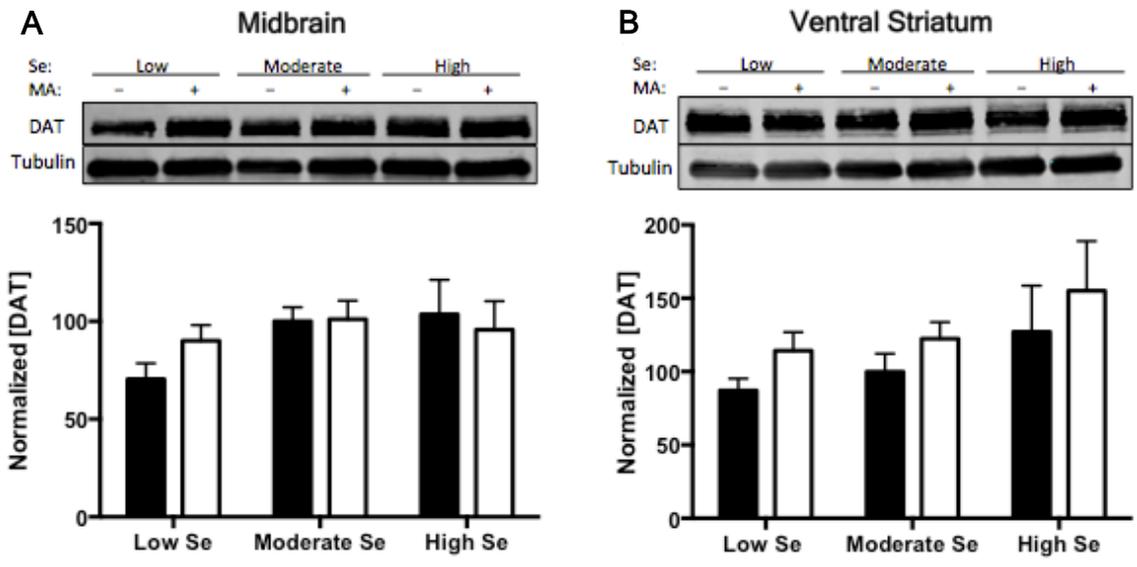


Figure 10. Effects of MA (5 x 5 kg/mg, i.p.) on DAT concentration in midbrain (A) and VSTR (B) of adult male and female C57BL/6 mice. Above: Representative western blot of DAT from animals raised on Se diet for 21 weeks before MA treatment. Bars show mean ± SEM for 5-6 animals/group. Graph of DAT protein mean ± SEM measured by optical density of western blot bands.

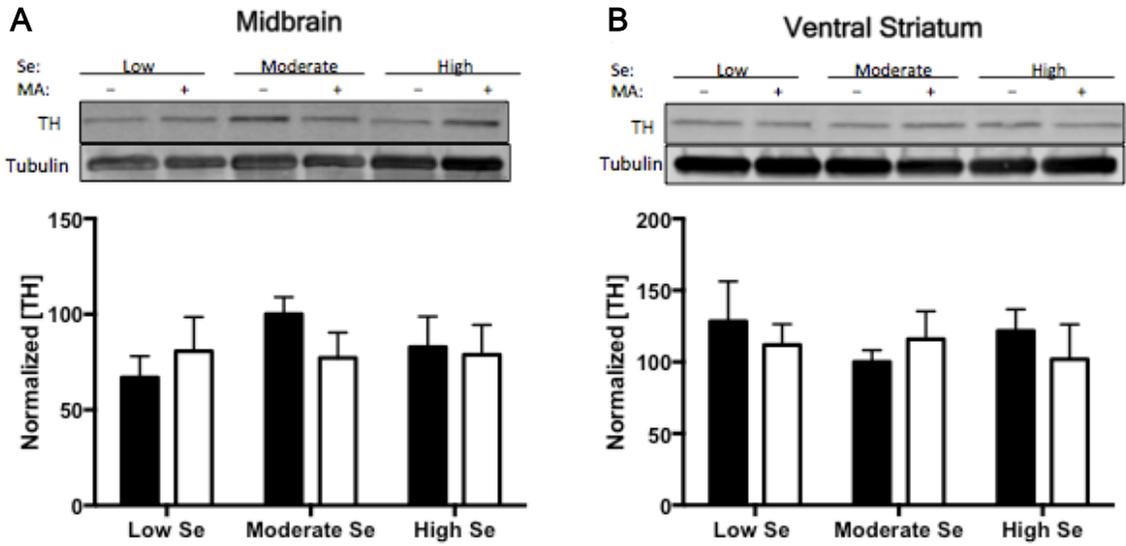


Figure 11. Effects of MA (5 x 5 kg/mg, i.p.) on TH concentration in midbrain (A) and VSTR (B) of adult male and female C57BL/6 mice. Above: Representative western blot of TH from animals raised on Se diet for 21 weeks before MA treatment. Bars show mean ± SEM for 5-6 animals/group. Graph of TH protein mean ± SEM measured by optical density of western blot bands.

CHAPTER VI

DISCUSSION and CONCLUSION

Previous studies report that Se protects against MA neurotoxicity and improves DA function [19, 20, 28, 29]. However, the mechanism by which Se protects against MA neurotoxicity has not been clarified. This study aims to identify which selenoproteins are involved in protecting against MA-induced neurotoxicity and to elucidate the mechanism by which these selenoproteins prevent neurodegeneration.

The first objective of this study was to identify individual selenoproteins that may be involved in neuroprotection against MA. We observed that the RNA of most selenoproteins increased significantly in 10 nM Se compared to Se-free media. Furthermore, protein concentrations increased in proportion to Se supplementation for all selenoproteins tested. The preliminary data suggested that there could be several selenoproteins that are important for neuroprotection. In addition, our data provides insight into the differential regulation of selenoproteins RNA and protein expression.

The second objective of the study was to determine if specific selenoproteins change expression with MA *in vitro*. Our results show that the selenoproteins GPX1 and GPX4 decrease in response to MA in differentiated cells, whereas GPX levels remained unchanged when challenged with MA in undifferentiated cells. This suggests that the decrease is dependent on DA synthesis. Moreover, GPX1 and GPX4 protein expression and enzymatic activity increased with increasing Se in media in SH-SY5Y cells, and decreased when challenged with 100 μ M MA for 24 h. In addition, MA was found to decrease tGSH, increase GSSG and increase ROS production at 0 nM Se, but not in the presence of 10 nM or 100 nM Se. These results suggest that Se supplementation limits MA-induced ROS production and prevents oxidative stress from MA by transferring the oxidation to glutathione. Furthermore, because GPX levels are much higher in Se supplemented conditions, the reduction by MA is still above adequate levels and therefore the decrease is less significant. Overall, these findings show the importance of Se in maintaining GPX levels as well as proper regulation of GSH in order to reduce MA-induced oxidative stress and increase protection against MA toxicity.

A study by Hom *et al.* reports that increasing expression of GPXs prevents increased ROS levels and lipid peroxidation caused by MA in PC12 cells, another dopaminergic neuronal model [28]. Our results support these findings by showing that MA-induced increases in ROS production at Se-depleted levels is prevented with Se supplementation. The reduction in ROS generation is likely due to the increased GPX1 and GPX4 protein expression. GPX1 and GPX4 contain Se in the form of Sec at the active site [22]. GPX1 protects cells against hydrogen peroxides and lipid hydroperoxides, whereas GPX4 functions to metabolize phospholipid

hydroperoxides found in the membrane [30]. Both GPXs enzymes protect SH-SY5Y cells from oxidative stress caused by ROS.

To further confirm that the GPX enzymes are protecting against MA-induced oxidative stress, we measured intracellular and extracellular tGSH content. GPXs transfer ROS to glutathione to reduce substrates. Within the cell, tGSH concentration was lower in Se-supplemented levels proposing that the GPX enzymes are utilizing the GSH to reduce ROS induced by MA. In addition, the ratio of intracellular GSSG to tGSH is higher at Se-supplemented levels, suggesting increased GPX activity due to higher expression. Our results show no significant changes in the extracellular GSSG fraction. These results agree with the reports of reduced tGSH from MA exposure in animal models [31]. There were no significant changes in the GSSG fraction relative to extracellular tGSH. However, high levels of tGSH were measured extracellular in 0 nM Se condition, suggesting that the cell is excreting GSH into the extracellular space. A study by Hirrliner *et al.* reports that the multidrug resistance protein 1 (Mrp1) mediates the release of GSH disulfide and GSH conjugates from rat astroglial cells [32]. Further investigation is needed to determine whether Mrp1 is involved in the export of GSH from dopaminergic neuronal cells when Se is low.

The third objective of our study was to determine changes in GPX enzymes and DA proteins in animal models. We analyzed the effect of MA on mice raised on either a low, moderate, or high Se diet on the function of dopaminergic neurons found within the mesolimbic dopaminergic system. Previous studies have investigated the effects of Se on dopaminergic neurons in the nigrostriatal dopaminergic pathway [33] and hippocampus. As DA primarily acts on the mesolimbic dopaminergic pathway, we measured changes in the midbrain and VSTR. The mesolimbic pathway is thought to regulate emotional behavior, reward, and motivation [34]. Dopaminergic neurons from the midbrain VTA project to the NAcc found within the VSTR. DA released in the NAcc modifies behaviors involved in motivation and reward seeking.

We observed an increase in GPX1 expression from low to moderate Se conditions in midbrain and an overall significant effect of Se between low and high Se conditions in VSTR. However, we did not observe any significant changes in GPX4 levels of the midbrain and VSTR. Interestingly, we observed a trend toward increased GPX1 and GPX4 in the presence of MA at low Se conditions, suggesting an increase in GPX enzyme synthesis in response to MA-induced ROS generation.

Overall, *in vivo* GPX levels were resistant to MA changes relative to what we observed *in vitro*. A possible explanation could be due to differences in the *in vitro* and *in vivo* cell population. Our cell culture experiments were conducted with a homogenous cell population, whereas mouse brain includes different types of neurons as well as other cells. GPX could be regulated differently in other cells, such as glial cells, present in the midbrain and VSTR. GPX4 is resistant to dietary Se deficiency [35], which could explain why we see a differential regulation between GPX1 and GPX4

To identify changes in DA neuronal integrity in response to MA, we measured DAT and TH levels as presynaptic markers of dopaminergic neurons. Previous reports show that acute and chronic exposure of MA in rodents decrease levels of DAT and TH [5] on striatal neurons. We did not observe significant changes in DAT and TH expression in response to Se and MA. A study by Ramos *et al.* reports that a low Se diet increases mRNA expression of TH and DAT in dopaminergic neurons within the nigrostriatal systems. In addition, studies have reported that the dopaminergic neurons of the nigrostriatal pathway is more sensitive to the neurotoxic effects of MA compared to the dopaminergic neurons in the mesolimbic pathway [36]. This difference could be reflected in the lack of changes in DAT and TH expression in the midbrain and VSTR. Another possible factor could be due to the MA regiment we used. Many rodent studies use acute exposures of multiple high doses of MA, which result in a similar degree of neurotoxicity seen in postmortem human studies [2]. However, we utilized a chronic MA regiment that was intended to mimic human patterns of MA use, which are normally based on years of exposure to the drug [2]. The length of our treatment (5 days) or the time period before sacrificing the mice (24 h) may not have been adequate to see any changes within the neurons.

In conclusion, our results demonstrate that dietary Se supplementation could provide protection against MA-induced oxidative stress by increasing GPX expression. This suggests that Se deficiency could exacerbate MA toxicity. MA is a known stimulant and the reported poor nutrition of MA users could exacerbate the effects of MA on the brain [37]. While Se levels in the US are generally adequate, a recent showed that poor African Americans have significantly lower Se levels than poor Caucasians [38], Thus some groups may be at greater risk of MA toxicity. These findings have important implications for treating patients with acute MA toxicity. Checking Se levels upon diagnosis of acute MA toxicity and maintaining Se levels by Se-supplementation may be an effective supplemental treatment. A better understanding of how MA affects other antioxidant proteins is necessary to develop treatments for the growing MA epidemic.

Future Studies

Future experiments may help further clarify the mechanism by which GPX enzymes protect against MA toxicity in animal models. Other studies would be to measure ROS concentrations and toxicity using TUNEL in midbrain and striatum. We could also look at GPX1 and GPX4 with immunohistochemistry along with neuronal and glial markers to see if there are different changes between the two cell populations. Elucidating the role of GPX1 and GPX4 in protection against MA-induced dopaminergic neurodegeneration will provide better insight into how dietary Se can be utilized clinically to treat MA users.

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