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# **The Effects of Aging and Anti-Aging Dietary Restriction on Brain Glutathione and Thioredoxin Redox Systems**

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## Supplementary text

### 1 Regulation of cellular cystine uptake and cysteine synthesis in astrocytes for GSH synthesis

#### *1.1 Decreased OTUB1 deubiquitinase persulfidation with brain aging may destabilize xCT to decrease astrocyte xc--mediated cystine uptake*

In cultured cells increased H<sub>2</sub>S generation was found to lead to increased persulfidation activating OTUB1, a deubiquitinase that stabilizes the cystine-glutamate antiporter (xc-) main subunit xCT (SLC7A11) [1, 2]. However, even though OTUB1 was found to be persulfidated in the brain of young adult mice, the level of OTUB1 persulfidation did not change significantly with dietary restriction (DR) [3]. There was, however, an aging-induced decline in mouse brain OTUB1 persulfidation, suggesting that increased xCT ubiquitination and degradation may contribute to decreased astrocyte xc--mediated cystine uptake in the aged brain [3]. But this was likely compensated for by increased SLC7A11 gene expression with aging [4, 5] or by increased astrocyte ASCT1-mediated cysteine uptake, as no change in mouse brain cysteine levels were found with aging [6].

Like persulfidation, glutathionylation was also shown to stimulate OTUB1 deubiquitinase activity to stabilize xCT. So, there may be a negative feedback loop regulating astrocyte glutathione (GSH) synthesis during DR, where the increased cytoplasmic GSH/glutathione disulfide (GSSG) during DR increases cytoplasmic glutaredoxin 1 (GLRX)-mediated deglutathionylation of OTUB1 to inhibit OTUB1 deubiquitinase activity leading to the increased ubiquitination and degradation of xCT decreasing xc--mediated cystine uptake for GSH synthesis [7].

#### *1.2 Astrocyte methionine cycle flux may be altered during fasting and DR*

In mammals the astrocyte homocysteine that potentially stimulates the transsulfuration pathway flux during fasting and DR may be derived in part from the methionine [8] released into the bloodstream from the liver [9] and skeletal muscle [10] as well as the methionine provided by the increased rate of autophagy and the decreased rate of protein synthesis occurring during these times. Fasting and DR may potentially stimulate methionine cycle flux, perhaps driven by increased expression of methyltransferase enzymes, leading to decreased S-adenosylmethionine (SAM) levels. SAM allosterically inhibits the methylenetetrahydrofolate reductase (MTHFR) enzyme that links the folate cycle to the methionine cycle and also allosterically activates the initial transsulfuration pathway enzyme cystathionine beta-synthase (CBS) (see Figure 1) [11]. So, the decreased SAM levels during fasting may increase flux into the methionine cycle to stimulate methylation reactions, but decrease flux through CBS. However, the increased brain H<sub>2</sub>S and protein persulfidation levels following 3 days of male mouse fasting [3] suggests a likely increase in astrocyte transsulfuration pathway flux, which is plausible even with decreased stimulation of CBS by SAM, since DR increased rat astrocyte CBS gene expression [12] and, in addition, cystathionine gamma-lyase (CTH), not CBS, is the rate-limiting step of the astrocyte transsulfuration pathway [13]. Another possibility is that the carboxyl-terminal Bateman module in mammalian CBS is proteolytically cleaved during DR leading to a constitutively active CBS enzyme similar to CBS orthologs in invertebrates that lack the Bateman module and thus are fully active in the absence of SAM [14].

During mouse fasting, increased beta-hydroxybutyrylation of methionine cycle enzyme S-adenosylhomocysteinase (AHCY) in liver was shown to decrease its enzyme activity [15] leading to increased S-adenosylhomocysteine (SAH) levels [16], although AHCY gene expression was increased [9]. However, beta-hydroxybutyrate levels in the brain may not rise high enough during fasting for AHCY

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beta-hydroxybutyrylation to occur. Surprisingly, unlike fasting for 16 to 48 hours, which increases mouse blood ketone body levels by roughly 5 to 20-fold depending upon the fast duration, age, and sex of the mice, with young female mice showing the largest increase [17], mice administered a DR diet only showed a 40% to 2-fold increase in the plasma levels of the ketone body beta-hydroxybutyrate, while an intermittent fasting (IF) diet only resulted in a 2 to 3-fold increase in plasma beta-hydroxybutyrate levels [18-20], suggesting that the beta-hydroxybutyrylation of brain AHCY and other proteins throughout the body are likely much less affected during DR or IF than during a single fast of roughly equal fasting duration.

*1.3 DR appears to have little effect on brain methionine cycle flux and DNA methylation in male mice, but there may be a sex-specific difference in female mice*

As of the writing of this article, no studies have been performed measuring the effects of fasting or DR on brain or isolated astrocyte methionine cycle and transsulfuration pathway flux. But indirect evidence suggests possible sex-specific differences. The fasting-induced increase in female mouse brain region-specific cysteine and GSH levels and decrease in methionine and homocysteine levels [21] suggest that the flux through these pathways could be increased in some female mouse brain regions. Likewise, the DR-induced increase in SAH levels in female, but not male, mouse brain also suggests that DR may stimulate astrocyte methylation reactions in female mice [22]. The DR-induced increase in SAH and other amino acids in female rodent brain appears to prevent the decreased mTOR expression that occurs during DR in the male rat brain that was associated with improved memory [23]. Male mice on a DR diet, on the other hand, did not show an altered total amount of global hippocampal CG or non-CG cytosine DNA methylation, although DR induced more hypermethylated than hypomethylated cytosines in DNA likely due to the DR-induced decreased gene expression of TET2 and TET3 demethylases, although expression of DNA methyltransferases DNMT1 isoform E1 and DNMT3A isoform 1 were also shown to decrease [24]. Another study confirmed that DR prevented the aging-related increase in mouse hippocampal DNMT3A levels [25]. DR was also shown to prevent 33% of the specific aging-related DNA methylation changes in a study of male C57BL/6 mouse hippocampus [24] and 83% of the aging-related DNA methylation changes in a study of female BALB/C mouse hippocampus [26].

In addition, male C57BL/6 mice administered an IF diet showed global hypomethylation of cerebral cortical DNA suggesting a possible decrease in cerebral cortical methionine cycle flux and SAM levels. But as a compensatory response, IF led to the increased levels of cerebral cortical DNA methyltransferases DNMT3A and DNMT3B and the decreased levels of the demethylase TET1 that led to an increase in the methylation of key promoter regions resulting in a balanced effect and increased cognitive function in the mice on the IF diet [27]. DR was also shown to prevent aging-related increases in the 5-methylcytosine immunoreactivity in DNA of male C57BL/6 mouse cerebellar Purkinje cells [28] and hippocampus [29]. So, fasting and DR did not stimulate astrocyte methionine cycle flux to induce global DNA hypermethylation in male mouse brain regions and even sometimes led to DNA hypomethylation.

In male mice, DR may instead increase the partitioning of the metabolism of astrocyte homocysteine away from the methionine cycle toward the transsulfuration pathway for increased H<sub>2</sub>S synthesis. Consistent with this hypothesis and the important role of increased transsulfuration pathway flux for increased neural function and longevity discussed below, male but not female rats administered an IF diet showed improved cognitive function [23]. DR or IF did not improve the cognitive function of aged outbred female mice [30], while a 40% DR diet increased the lifespan of male but not female C57BL/6 mice [31]. Consistent with these results, every-other-day (EOD) feeding initiated late in life (20 months)

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was shown to decrease hypothalamic proinflammatory gene expression and frailty in male, but not female mice, while male mice on the IF diet also showed increased cognitive function [32, 33].

Long-term fasting of male and female elephant seals was shown result in global DNA hypermethylation of blood cells [34], which we also hypothesize may occur in specific brain regions of female mice on a DR diet, likely stimulated by increased methionine cycle flux. Future studies should, however, also focus on the hypothalamus as it signals to white adipose tissue [35] through the sympathetic nervous system to regulate cellular senescence and systemic inflammation potentially impacting the rate of aging [36].

The decrease in SAM levels in the brain during fasting is likely blunted compared to that in the liver in part due to the brain using the methionine adenosyltransferase 2A (MAT2A) enzyme for SAM synthesis (Table S1), while the adult liver primarily uses the methionine adenosyltransferase 1A (MAT1A) enzyme. Mouse liver MAT1A levels increased with fasting or DR increasing methionine cycle flux and the rate of methylation reactions [16, 37]. MAT2A has a lower  $K_m$  for methionine binding, with the enzyme being saturated at physiological methionine levels, and is much more sensitive to feedback inhibition by SAM than MAT1A. So MAT2A is fine-tuned to maintain stable lower levels of SAM in brain than those normally found in the liver. In liver during times of stress, SAM levels can fluctuate markedly, such as shown by the large decrease observed during fasting [38-40]. MAT1A or MAT2A is the rate limiting step of the methionine cycle. But since the methionine cycle and transsulfuration pathway compete for the substrate homocysteine, the increased astrocyte transsulfuration pathway flux during DR in male mice may decrease flux through the methionine cycle. The brain of long-lived Ames dwarf mice showed no change in the transmethylation flux through the methionine cycle compared to normal control brain, but the liver of Ames dwarf mice showed increased transmethylated flux [41].

### **2 Fasting increases the levels of select metabolites in the blood**

#### *2.1 Branched chain amino acids (BCAAs), 2-hydroxybutyrate (2-HB), and 2-ketobutyrate (2-KB)*

Fasting for 2 to 7 days in humans robustly increased plasma levels of the three branched-chain amino acids (BCAAs) [42, 43] (Table 1). In contrast, male C57BL/6J mice fasted for 2 days did not show altered BCAA levels in the serum (Table 2), while the levels of most other amino acids declined [44]. In rats, a 3 day fast increased plasma isoleucine and total BCAA levels, while leucine and valine levels did not significantly increase [45]. Fasting increased the levels of the three BCAAs in the female C57BL/6 mouse brain [21, 46]. During fasting, transsulfuration pathway product alpha-ketobutyrate (2-ketobutyrate (2-KB)), which is also a product of threonine catabolism, together with alpha-hydroxybutyrate (2-hydroxybutyrate (2-HB)), the product of the lactate dehydrogenase-mediated reduction of 2-KB, accumulated to high levels in plasma [47]. 2-HB was shown to inhibit the catabolism of BCAAs by inhibiting branched chain aminotransferase 2 (BCAT2) [48] contributing to the increased BCAA levels during fasting. 2-KB systemically accumulates during fasting not only due to increased hepatic transsulfuration pathway flux, but also due to its slowed catabolism through the fasting-mediated inhibition of branched chain ketoacid dehydrogenase (BCKDH) and pyruvate dehydrogenase (PDH) complexes [48] in tissues such as the liver and the hypothalamus [49]. but PDH and BCKDH are not inhibited by fasting in the rest of the brain [50, 51]. Fasting also induced the accumulation of 2-HB in female mouse brain [21] and in the short term this inhibited BCAA catabolism leading to mouse brain BCAA accumulation. But over longer periods of fasting, 2-HB was shown to activate the transcriptional regulator C/EBP $\beta$  to induce the expression of BCAA catabolic enzymes leading to their breakdown for energy generation [48], potentially explaining the transient accumulation of mouse brain BCAAs during fasting.

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## 2.2 2-aminobutyrate (2-AB) and ophthalmic acid

Like measurements of the pyruvate/lactate, tissue or blood 2-KB/2-HB can be measured as an indicator of the cytoplasmic [NAD<sup>+</sup>]/[NADH] [52]. 2-KB can be transaminated using glutamate as an amino donor to form 2-aminobutyrate (2-AB), an analog of cysteine. During fasting, when 2-AB plasma concentrations greatly rise [47], and other times of cysteine limitation, cysteine-glutamate ligase (CGL) increasingly catalyzes the reaction of 2-AB, instead of cysteine, with glutamate. Glutathione synthetase (GSS) also readily uses gamma-glutamyl-2-aminobutyrate for the reaction with glycine to form the GSH analog ophthalmic acid, an important signaling molecule regulating GSH metabolism [53] as well as a potential neurotransmitter in the brain stimulating motor function [54].

## 2.3 Taurine

The effects of fasting on the plasma and brain levels of the sulfur amino acids (SAAs) cysteine, cystine, methionine, and taurine are not very well-established, although human plasma taurine levels were shown to increase following 3 days of fasting [55] but decline following 7 days of fasting [56]. Taurine is an abundant amino acid linked to many health benefits, including longevity [57], which is present in the diet and also synthesized endogenously, primarily by the liver, from cysteine using the cysteine dioxygenase 1 (CDO1), cysteine sulfinic acid decarboxylase (CSAD), and flavin monooxygenase 1 (FMO1) enzymes [58]. The human brain was shown to take up less taurine with aging [59]. Taurine cannot be used as a source of cysteine for GSH synthesis, so excess taurine is either conjugated to bile acids or is excreted in the urine. However, taurine has been shown to increase brain GSH levels during times of oxidative stress by preserving the levels of antioxidant enzymes such as glutathione reductase (GSR) [60].

## 3 Expression changes of superoxide dismutases (SODs) and catalase (CAT) with aging and DR

### 3.1 Changes in SOD1, SOD2, and SOD3 gene expression with aging:

Studies of 4 male Sprague-Dawley SD rat brain regions found that the gene expression of cytoplasmic superoxide dismutase 1 (SOD1) increased by 40% with aging in the cerebellum, increased by 15% with aging in the hippocampus, and decreased by 15% with aging in the striatum, while there was no change in expression with aging in the parietal cortex [61]. However, male Sprague-Dawley rat SOD1 protein levels increased with aging in both the hippocampus and striatum [62]. In brain from male C57BL/6N mice, SOD1 activity was shown to decline by 21% from 12 to 24 months of age [63]. Another study found a larger decrease in the expression of brain SOD1 with aging in female than male C57BL/6 mice [64]. Unlike C57BL/6 mice, C57BL/6 x CBA/6J mice showed increased brain SOD1 activity with aging [65]. So, changes in brain SOD1 expression and activity with aging are variable across brain regions and rodent strains.

ScRNA-seq data from young adult mouse brain showed that SOD1 gene expression was extremely high in ependymal cells, very high in endothelial cells, moderately high in astrocytes and fibroblast-like cells, and moderate in neurons, oligodendrocytes, and microglia. Mitochondrial superoxide dismutase 2 (SOD2) gene expression was shown to be very high in mouse astrocytes and ependymal cells, moderately high in endothelial cells, and moderate in neurons, oligodendrocytes, microglia, and polydendrocytes. Gene expression of mouse superoxide dismutase 3 (SOD3), which is a secreted enzyme [66], was shown to be moderately high in fibroblast-like cells, moderate in astrocytes, and fairly low in other neural cell types (Table S1) [67]. A bulk RNA-seq study of different mouse brain regions showed that SOD1 was

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differentially expressed with aging in the choroid plexus, while SOD2 gene expression decreased with aging in the hypothalamus, cerebellum, and combined caudate and putamen [40].

A 2014 review reported that DR only increased brain SOD activity in 4 of 23 measurements, with the rest of the measurements showing no change in activity [68]. But 40% DR was shown to increase Sprague-Dawley rat brain mitochondrial SOD2 activity [69]. In addition, DR was shown to lead to the differential expression of SOD3 in the mouse SVZ [67, 70].

### *3.2 Decreased expression of CAT in several neural cell types with mouse brain aging:*

CAT activity was measured in isolated neurons, astrocytes, oligodendrocytes, and microglia from young adult rats and found to be highest in oligodendrocytes and lowest in microglia [71]. CAT gene expression in the adult mouse brain was roughly 5-fold lower than SOD1 and at a similar level as SOD2. Mouse CAT was shown to be expressed at very high levels in astrocytes, moderate levels in endothelial cells, fibroblast-like cells, oligodendrocytes, ependymal cells, and microglia, and at low levels in neurons [72].

ScRNA-seq analysis of mouse brain showed that in almost all brain regions CAT gene expression decreased with aging in oligodendrocytes, neural endothelial cells, pericytes, and vascular leptomeningeal fibroblast-like cells (VLMCs), while in many brain regions CAT gene expression decreased with aging in oligodendrocyte precursor cells (OPCs) and vascular smooth muscle cells (VSMCs). Genes involved in the response to reactive oxygen species (ROS) declined with aging in mouse brain excitatory neurons, neural endothelial cells, VLMCs, and neural VSMCs [73]. The steady decrease in CAT gene expression with aging in oligodendrocytes may contribute to the increased aging rate of white matter tracts [73]. CAT was shown to be differentially expressed with aging in the mouse olfactory bulb [67]. The decreased CAT expression with aging puts more burden on the NADPH-dependent glutathione peroxidase (GPX)-GSH-GSR and peroxiredoxin (PRDX)-thioredoxin (TXN)-TXN reductase (TXNRD) systems for H<sub>2</sub>O<sub>2</sub> detoxification.

### *3.3 DR increased CAT persulfidation in the brain*

In the brain of mice on a DR diet, there was a 60-fold increase in CAT persulfidation levels, the fourth highest increase of any protein measured in the persulfidome [3]. In *Arabidopsis* CAT persulfidation was shown to decrease its enzymatic activity [74]. Most DR studies, with two exceptions stated in the 2014 review found that DR in rodents did not alter SOD or CAT activity in the brain [68]. The authors reported that DR only increased the activity of brain CAT in 6 of 22 measurements, with the rest of the measurements showing no change in CAT activity [68]. The exceptions included male Fischer 344 rat cerebellar CAT activity declining with aging that was reversed by DR [75] and female BALB/c mouse cerebral hemisphere CAT activity declining with aging that was reversed by DR [76]. Two more recent studies using rabbits or male Sprague-Dawley rats even showed that IF decreased brain CAT activity [77, 78]. However, in other recent studies, DR was shown to increase hippocampal CAT gene expression in male C57BL/6JN mice [79], while IF was shown to increase hippocampal CAT and SOD2 protein levels in gerbils [80], once again supporting the important role of DR and IF in increasing hippocampal antioxidant enzyme levels.

## **4 Expression changes of glutathione disulfide reductase (GSR) with aging and DR**

In liver the cytoplasmic [NADPH]/[NADP<sup>+</sup>] has been shown to be present at a similar ~ 100:1 ratio as the cytoplasmic GSH/GSSG [81]. However, in some transformed cell lines in culture the cytoplasmic [NADPH]/[NADP<sup>+</sup>] was shown to be only roughly 1:1, providing little reductive power for GSR to reduce GSSG to GSH and perhaps even allowing some reversal of GSR flux during acute oxidative

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stress for the reduction of NADP<sup>+</sup> by GSH [82]. The steady-state cytoplasmic [NADPH] (~ 3-10 μM) in cultured cells is approximately 1,000-fold less than the cytoplasmic GSH concentration (~ 3-10 mM) that it regulates. In most cells and tissues, but not in the heart, the mitochondrial GSH/GSSG was shown to be more reduced when compared to the cytoplasmic GSH/GSSG [81, 83]. However, there are a few studies that found that the GSH/GSSG in the mitochondrial matrix [84] and cytoplasm [85] to have roughly equal redox potentials. The activities of cytoplasmic NADPH-generating enzymes fueling GSR function were shown to be higher in young adult female than male Sprague-Dawley rat cerebral cortex [86].

GSR activity was shown to decrease by 4-fold in the rat cerebral cortex during the first 2 months after birth [87]. Aging from 4 to 17 months increased male and female Sprague-Dawley rat brain GSR activity by 20% to 50% in the cerebral cortex, midbrain, striatum, and cerebellum [88]. Another study found Sprague-Dawley rat brain GSR activity to increase by 40% from 16 to 24 months of age [89], while another study found male Hooded-Lister rat brain GSR activity to increase by 25% from 1 month to 17 months of age [90]. However, other groups found no change in male Sprague-Dawley rat brain GSR activity from 1 month to 9 months of age [91] or in male Fischer 344 rats from 4 months to 26 months of age [92], while another study found decreased male Wistar rat brain GSR activity with aging from 4 to 24 months of age in the brainstem, cerebral hemispheres, cerebellum, and diencephalon [93]. So, changes in rat brain GSR activity with aging are highly dependent on the particular rat strain studied. Both male Sprague-Dawley and Wistar rats have reported mean lifespans of 29.5 months compared to 26 months for male Fischer 344 rats [94]. So, mean lifespan does not strongly correlate with aging-related changes in brain GSR activity.

A detailed study was performed measuring GSR activity in 4 male Wistar rat brain regions over 7 time points across the lifespan. In that work cerebral cortical GSR activity was shown to be stable from 5 months until 15 months of age, after which it declined until 35 months of age. In the combined caudate and putamen GSR activity was shown to increase from 5 to 20 months of age, after which it declined until 35 months of age. In the substantia nigra, GSR activity was shown to remain constant from 5 to 15 months of age, increase from 15 until 25 months of age, and then decrease from 25 until 35 months of age. Lastly, in the thalamus GSR activity was shown to increase from 5 to 15 months of age and then decrease from 15 to 35 months of age. In each brain region the decrease in GSR activity in later adulthood was larger than the increase during early adulthood, summing to decreased GSR activity with aging [95], consistent with the Wistar rat GSR activity study described in the preceding paragraph [93].

Following 4 weeks of DR that was initiated at 18 months of age, there was differential gene expression of GSR in the mouse choroid plexus [67]. One study found that administering a 40% DR diet increased GSR activity in whole brain from Sprague-Dawley rats [69]. Another study showed that 40% DR for 11 months increased GSR activity in aged male Sprague-Dawley rat hippocampus, striatum, and cerebellum, but not in the parietal cortex. DR resulted in a parallel increase in GSH levels in these same rat brain regions [61]. In another study of middle-aged male Sprague-Dawley rats, long-term DR when combined with endurance swim exercise decreased cerebral cortical GSR activity and GSH levels, perhaps in part due to an exercise-induced increase in H<sub>2</sub>O<sub>2</sub> generation [96] depleting GSH leading to oxidative damage [97]. A study using male Hooded-Lister rats consuming a 30% DR diet found no effect of the diet on brain GSR activity when assayed at 17 months of age [90], which may be due to the minimal effects of DR on cerebral cortical GSR activity as described above or due to a rat strain-dependent effect. Lastly, a study using male Wistar rats on a 15% DR diet found no effect of the DR diet on hippocampal GSR protein levels or activity [98].

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## 5 Changes in glutathione peroxidase 1 (GPX1) expression and activity with aging and DR

Two studies using rats showed decreased brain GPX1 gene expression with aging. For example, one study found Sprague-Dawley rat brain GPX activity to decrease with aging by 30% from 16 to 24 months of age [89]. Another study showed decreased GPX activity in four brain regions of 24-month old male Wistar rats compared to that of 12-month old controls [93]. However, one study found GPX activity to increase by 13% to 50% in male and female Sprague-Dawley rats with aging from 4 to 17 months in the cerebral cortex, striatum, midbrain, and cerebellum [88]. In a study of GPX activity in different brain regions of male Wistar rats, GPX activity increased in the cortex from 5 to 30 months of age, increased in the caudate and putamen from 5 to 25 months of age, and decreased in separate thalamic and substantia nigral fractions from 10 to 25 months of age [95]. So, the aging-induced changes in enzyme activity appear to be brain-region specific, but inconsistent findings between groups limit our current understanding of these changes.

Several studies using mice found no change in brain GPX1 activity with aging. For example, in male C57BL/6 mice GPX1 activity did not change with aging [63]. In humans and most rat strains, females have a slightly longer lifespan than males. But in C57BL/6 mice, males can be slightly longer-lived and this was suggested to be caused by the decreased expression of GPX1 in the female brain with aging [64]. Like C57BL/6 male mice, C57BL/6 x CBA/6J hybrid male mice also showed no change in brain GPX1 activity with aging [65]. However, one study using C57BL/6J mice found that GPX activity in the cerebral cortex declined 2-fold in females from 6 to 18 months of age and declined 3-fold in males during this time [99]. In addition, a study of male C57BL/6N mice found GPX activity to increase from 6-months to 24 months of age in the frontal cortex, hippocampus, caudate nucleus, and brainstem, but not in the cerebellum [100].

The authors of a 2014 review [68] reported that of eight studies measuring the effects of DR on GPX activity in the brain, DR had no effect on GPX activity in five studies [75, 90, 101-103], increased GPX activity in two studies [101, 102], and decreased GPX activity in one study [75]. We found two more studies where DR was shown to increase brain GPX activity. In the first, 40% DR was shown to increase GPX activity in Sprague-Dawley rat brain [69]. In the second, a long-term DR diet was shown to increase GPX activity in the hippocampus and parietal cortex, but not in the striatum or cerebellum of rats. DR increased total thiols in the hippocampus, parietal cortex and cerebellum, but not in the striatum, while DR increased GSH levels in the hippocampus, striatum, and cerebellum, but not in the parietal cortex. The GSH results paralleled the results of DR on GSR activity, where DR increased GSR activity in other brain regions but not in the cortex [61]. We also found two more studies where DR decreased brain GPX activity. One study reported that when long-term DR was combined with endurance exercise it resulted in decreased rat cerebral cortical GPX activity [97]. In another study, DR administered to Wistar rats for 3-months decreased mitochondrial ROS and GPX activity in the cerebral cortex and hippocampus and led to increased GSH levels in both brain regions [104].

## 6 Monothiol glutaredoxin 3 (GLRX3) and glutaredoxin 5 (GLRX5)

As monothiol glutaredoxins, GLRX3 and GLRX5 do not typically participate in glutathionylation or deglutathionylation thiol-disulfide exchange reactions. GLRX3 is targeted to the cytoplasm [105] and nucleus [106], while GLRX5 is targeted to the mitochondrial matrix [107]. GLRX3 knockout was shown to be embryonic lethal in mice, likely due to its essential function in hemoglobin maturation, hematopoiesis, and cell cycle progression [108]. GLRX5 knockout mice developed a severe sideroblastic anemia due to the essential role of GLRX5 in hemoglobin synthesis and iron-sulfur cluster

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biosynthesis [109]. DR was shown to cause a 5-fold increase in GLRX5 persulfidation in mouse brain, while EOD feeding increased GLRX3 persulfidation by 2-fold [3].

### **7 Neural cell type gene expression of redox enzymes**

#### *7.1 Brain cell type gene expression of TXN and TXNRD genes:*

ScRNA-seq studies of adult mouse brain showed that TXN reductase 1 (TXNRD1) was expressed fairly uniformly in different neural cell types with highest levels in polydendrocytes, fibroblast-like cells, and ependymal cells followed by neurons, endothelial cells and astrocytes with lowest expression in microglia and oligodendrocytes (Table S1) [110]. Mouse mitochondrial TXN reductase 2 (TXNRD2) was shown to be expressed at low levels in the mouse brain with moderately low expression in microglia, low expression in astrocytes, and very low expression in the other neural cell types. TXN reductase 3 (TXNRD3) has a primarily nucleocytoplasmic, but also a low level mitochondrial localization [111], and not only possesses TXNRD activity but also possesses low level GSR activity and glutaredoxin activity [112]. TXNRD3, most highly expressed in sperm [112], was shown to be expressed weakly in the mouse brain at a level roughly 5-fold lower than TXNRD1. TXNRD3 was shown to be expressed at low levels in brain fibroblast-like cells, ependymal cells, and endothelial cells, and at very low levels in the other neural cell types (Table S1) [110].

Nucleocytoplasmic TXN was shown to have very high expression in mouse brain, especially in ependymal cells, endothelial cells, and fibroblast-like cells, with moderately high expression in polydendrocytes, neurons, and astrocytes, and moderate expression in microglia and oligodendrocytes. Mitochondrial TXN2 was shown to have a relatively uniform moderate expression pattern in the mouse brain with highest expression in astrocytes, endothelial cells, and fibroblasts, and slightly lower expression in polydendrocytes, neurons, oligodendrocytes, and microglia. Thioredoxin interacting protein (TXNIP) was shown to have high expression in microglia, ependymal cells, fibroblast-like cells, and endothelial cells, with moderately low expression in astrocytes and polydendrocytes, low expression in oligodendrocytes, and almost no expression in neurons (Table S1) [110].

#### *7.2 Brain cell type gene expression levels of GPX genes:*

ScRNA-seq data of GPX gene expression from adult mouse brain are shown in Table S1 [110]. The data showed very high GPX1 expression in endothelial cells and microglia and moderately high expression in fibroblast-like cells and ependymal cells with moderate expression in other cell types [72]. GPX3 showed moderate expression in microglia, moderately low expression in ependymal cells, and low expression in other neural cell types [110]. GPX4 was expressed at moderate levels in oligodendrocytes, ependymal cells, and endothelial cells, slightly lower levels in fibroblast-like cells, and even lower levels in microglia and astrocytes, with the lowest expression being found in polydendrocytes and neurons. There was moderate GPX7 expression in fibroblast-like cells and endothelial cells, with lower expression in polydendrocytes, astrocytes and ependymal cells, and with very low expression in microglia, neurons, and oligodendrocytes. GPX8 showed modestly high expression in fibroblast-like cells and ependymal cells and moderate expression in astrocytes, with low expression in polydendrocytes, and very low expression in endothelial cells, microglia, oligodendrocytes, and neurons [110].

#### *7.3 Brain cell type gene expression levels of PRDX genes:*

ScRNA-seq data of PRDX gene expression from adult mouse brain are shown in Table S1 [110]. PRDX1 showed extremely high expression in oligodendrocytes and astrocytes with next highest expression levels in endothelial cells, fibroblast-like cells, ependymal cells, and polydendrocytes, with moderately

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high expression in microglia, and with only moderate expression in neurons. PRDX2 showed high expression in the mouse brain, with levels being highest in polydendrocytes and astrocytes, followed by neurons, ependymal cells, endothelial cells, and fibroblast-like cells, and with oligodendrocytes and microglia only having moderately high expression. PRDX3 was shown to be expressed relatively uniformly at moderate levels throughout neural cells with just slightly higher expression in astrocytes than neurons and oligodendrocytes and just slightly lower expression in microglia. PRDX4 was shown to be moderately expressed in nearly all neural cell types throughout the mouse brain with high expression in ependymal cells, moderately high expression in fibroblast-like cells, and moderate expression in endothelial cells, astrocytes, microglia, and oligodendrocytes, but with moderately low expression in neurons. PRDX5 was shown to be highly expressed in the mouse brain, at roughly 4-fold higher levels than PRDX3, and was expressed most highly in neurons, microglia, and fibroblast-like cells followed by astrocytes and ependymal cells and was only moderately expressed in oligodendrocytes and endothelial cells. PRDX6 was shown to have extremely high expression in astrocytes and ependymal cells and moderate expression in other neural cell types throughout the adult mouse brain. Compared to the cell types showing very high PRDX6 expression, PRDX6 expression was roughly 8-times lower in oligodendrocytes and roughly 10-times lower in microglia and neurons, but it was still moderately expressed in these cell types [110].

### ***7.4 Brain cell type gene expression levels of glutaredoxin genes:***

ScRNA-seq data of the expression of the 4 glutaredoxin genes from adult mouse brain are shown in Table S1 [110]. GLRX was shown to be expressed at low levels in the brain with highest gene expression in ependymal cells and astrocytes and moderately low expression in neurons, microglia, and endothelial cells, and lowest gene expression in oligodendrocytes. GLRX2 was shown to have highest gene expression in astrocytes, polydendrocytes, oligodendrocytes, neurons, and endothelial cells, with moderate expression in ependymal cells, fibroblast-like cells, and microglia [110]. GLRX3 was shown to have highest expression in endothelial cells and polydendrocytes with slightly lower expression oligodendrocytes, astrocytes, microglia, fibroblasts, and neurons, with lowest, but still modest expression in ependymal cells. GLRX5 was shown to have a similar gene expression pattern, but slightly higher expression than GLRX3, with highest expression in polydendrocytes and endothelial cells and modest expression in all other neural cell types (Table S1) [110].

### ***7.5 Brain cell type gene expression levels of glutathione-S-transferase (GST) genes:***

ScRNA-seq data of GST gene expression from adult mouse brain are shown in Table S1 [110]. GSTA4 was shown to be highly expressed in endothelial cells and ependymal cells with moderate expression in astrocytes and fibroblast-like cells, moderately low expression in neurons, and low expression in microglia, polydendrocytes, and oligodendrocytes. GSTK1 was shown to be expressed most highly in astrocytes with moderate expression also in fibroblast-like cells, endothelial cells, and oligodendrocytes, with low expression in polydendrocytes, and microglia, and almost no expression in neurons. GSTM1 was shown to be extremely highly expressed in mouse astrocytes and ependymal cells, with very high expression in fibroblast-like cells and endothelial cells, moderate expression in oligodendrocytes and microglia, and low expression in polydendrocytes and neurons. GSTM2 was shown to be expressed at moderate levels in fibroblast-like cells, moderately low levels in endothelial cells, low levels in ependymal cells, microglia, and polydendrocytes, and with almost no expression in astrocytes, neurons, or oligodendrocytes. GSTM4 was shown to be expressed at low levels in all neural cells, but at slightly higher levels in ependymal cells. GSTM5 was shown to be expressed at very high levels in astrocytes and oligodendrocytes, with moderately high expression in polydendrocytes and ependymal cells, and

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moderate expression in the other neural cell types. GSTM6 was shown to be expressed at very low levels in all neural cell types with highest expression being in ependymal cells, oligodendrocytes, and astrocytes. GSTM7 was shown to be expressed at moderately high levels in oligodendrocytes, moderate levels in endothelial cells, moderately low expression in ependymal cells, fibroblast-like cells, and polydendrocytes, and very low expression in microglia and neurons (Table S1) [110].

GSTO1 was shown to be expressed at high levels in polydendrocytes, at moderately low levels in oligodendrocytes, neurons, and microglia, and at low levels in astrocytes, ependymal cells, endothelial cells, and fibroblast-like cells. GSTO2 was shown to be expressed at low levels in ependymal cells and at very low levels in other neural cell types. GSTP1 was shown to be expressed at very high levels in oligodendrocytes, at moderate levels in fibroblast-like cells, endothelial cells, ependymal cells, and polydendrocytes, and at low levels in astrocytes, microglia, and neurons. GSTP2 was shown to be expressed at very low levels in all neural cell types. GSTT1 was shown to be expressed at moderate levels in fibroblast-like cells, slightly lower levels in ependymal cells and astrocytes, low levels in polydendrocytes, and endothelial cells, and was expressed at very low levels in microglia, neurons, and oligodendrocytes. GSTT2 was shown to be expressed at moderate levels in endothelial cells and fibroblast-like cells, at low levels in astrocytes, ependymal cells, and microglia, and hardly expressed in polydendrocytes, oligodendrocytes, or neurons. GSTT3 was shown to be expressed at low levels in fibroblast-like cells, astrocytes, and endothelial cells, and at very low levels in microglia and ependymal cells, and hardly expressed in oligodendrocytes, polydendrocytes, or neurons. GSTZ1 was shown to be expressed at moderate levels in astrocytes, moderately low levels in ependymal cells, oligodendrocytes, polydendrocytes, neurons, and fibroblast-like cells, and at low levels in endothelial cells and microglia. MGST1 was shown to be expressed at moderately high levels in astrocytes, ependymal cells, fibroblast-like cells, and endothelial cells at low levels in microglia, polydendrocytes, and oligodendrocytes, with little expression in neurons. MGST3 was shown to be expressed at moderate levels in neurons, endothelial cells, and oligodendrocytes, at moderately low levels in microglia, polydendrocytes, astrocytes, and fibroblast-like cells, and at low levels in ependymal cells (Table S1) [110].

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## SUPPLEMENTARY DATA

**Supplementary Table 1.** Median cell type expression of genes affecting redox function and metabolism in adult mouse brain (transcripts per 100,000 in cluster). Data taken from <http://dropviz.org> [12].

Gene	Astrocytes	Endothelial cells	Ependymal cells	Fibroblast-like cells	Microglia	Neurons	Oligodendrocytes	Polydendrocytes
SOD1	37.0	56.0	105.0	37.0	24.0	30.0	22.0	21.5
SOD2	18.0	9.0	18.5	7.2	7.0	10.0	8.2	11.5
SOD3	7.2	1.4	1.8	31.0	1.6	0.2	0.3	0.4
CAT	17.0	8.5	7.5	8.5	6.0	2.0	8.5	13.0
GCLC	5.2	3.1	18.8	4.3	6.2	6.5	3.8	4.2
GCLM	17.8	20.1	21.5	6.3	7.2	9.3	18.7	10.9
GSS	1.7	1.6	1.8	1.4	1.6	1.4	7.9	4.2
SIRT2	21.1	13.1	21.8	11.3	13.8	7.3	96.0	310.0
GSR	3.2	4.8	11.5	2.8	3.2	3.0	3.3	3.8
TXNRD1	4.8	5.8	7.5	7.8	4.3	6.8	4.0	9.5
TXNRD2	1.8	0.8	1.2	1.2	3.1	1.4	1.3	1.3
TXNRD3	1.1	1.7	2.2	2.5	0.8	0.6	0.4	0.6
TXN	17.0	45.0	80.0	35.0	14.0	18.0	12.0	19.0
TXNDC17	8.7	26.0	6.1	17.6	11.8	4.6	17.1	12.2
TXN2	13.0	13.0	6.2	12.0	8.0	9.0	8.0	10.0
GLRX	3.2	2.0	3.5	0.9	2.0	2.2	0.4	1.4
GLRX2	15.0	12.0	8.5	8.5	7.3	12.5	13.0	15.0
GLRX3	6.9	9.7	4.0	5.9	6.2	5.8	7.1	9.3
GLRX5	9.1	12.2	9.9	8.7	8.1	9.2	9.3	16.4
PRDX1	90.0	70.0	50.0	58.0	34.0	12.0	100.0	50.0
PRDX2	37.0	24.0	27.0	24.0	16.0	27.0	17.0	53.0
PRDX3	7.5	6.0	7.2	7.5	5.0	5.5	6.2	6.0
PRDX4	8.0	10.0	28.0	16.0	6.5	1.6	5.0	6.0
PRDX5	22.5	12.0	22.0	27.0	28.0	33.0	13.5	17.0
PRDX6	160.0	12.0	110.0	16.0	9.0	4.9	17.0	10.0
GPX1	6.7	56.0	16.0	27.0	42.0	7.2	6.5	11.5
GPX3	0.4	0.1	2.0	0.5	6.0	0.5	0.2	0.6
GPX4	3.8	6.5	7.5	5.5	4.0	2.2	7.5	2.8
GPX6	0.05	0.2	0.1	0.2	0.4	0.1	0.05	0.1
GPX7	2.6	5.4	1.7	8.2	0.5	0.4	0.3	3.8
GPX8	6.5	0.7	19.5	21.5	0.6	0.2	0.3	2.1
GSTO1	2.3	1.1	1.5	0.9	3.0	3.5	4.2	33.0
GSTO2	0.4	0.1	1.4	0.4	0.3	0.1	0.7	0.5
GSTP1	4.5	8.0	7.8	11.5	3.8	2.6	52.0	7.5
GSTP2	0.0	0.1	0.0	0.4	0.3	0.0	0.2	0.1
MEF2C	4.7	23.8	2.5	9.6	104.0	53.0	3.0	3.5
G6PD	3.5	1.6	1.0	2.4	4.2	2.5	3.2	2.8
PGLS	7.0	10.1	9.8	8.5	9.8	2.4	5.6	8.0
PGD	3.3	2.5	3.0	10.0	5.5	2.4	2.5	4.0
ME1	9.0	0.8	1.0	1.3	1.2	3.0	2.5	5.0
IDH1	12.0	3.0	4.2	4.2	4.5	2.4	8.0	25.0
IDH2	11.0	3.4	31.0	8.5	14.0	1.8	3.1	4.2
ALDH1L1	34.0	0.7	1.6	1.7	1.3	0.4	1.0	0.4
GFAP	41.0	0.6	0.9	0.4	0.8	0.4	1.4	0.5
MTHFR	0.3	0.7	1.4	1.5	2.2	1.3	0.2	1.3
GAPDH	1.4	0.5	0.6	0.5	0.7	1.3	0.7	0.6
ALDOA	65.0	31.5	30.8	49.0	37.5	108.0	44.6	27.3

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PGK1	1.7	0.7	2.7	0.7	0.7	1.3	1.3	1.6
NFE2L1	15.8	45.0	17.5	42.0	18.5	45.0	12.5	17.2
NFE2L2	18.5	16.7	6.5	16.5	23.0	0.3	6.9	3.2
NFE2L3	0.1	0.3	0.1	0.4	0.4	0.2	6.8	3.2
KEAP1	7.4	12.4	7.4	8.7	7.9	8.8	8.3	8.2
CUL3	9.5	10.9	9.4	10.8	10.0	16.8	7.2	7.9
BACH1	1.3	5.5	0.8	5.3	8.1	1.2	2.7	3.5
BACH2	2.5	1.3	1.1	0.7	3.2	2.1	1.6	3.9
CYBB	0.05	0.1	0	0.1	17.5	0.05	0.05	0.1
FOXO1	13.2	15.3	17.2	7.4	4.5	1.7	2.7	1.8
FOXO3	4.2	2.7	2.5	4.2	4.0	4.6	1.7	2.3
ATF4	11.0	12.0	3.5	16.0	14.0	17.0	7.5	15.5
CBS	13.0	0.2	5.5	0.7	0.5	0.2	0.7	0.3
CTH	4.3	0.1	0.2	0.3	0.3	0.2	0.2	0.3
MPST	3.9	0.5	4.0	1.4	0.9	0.6	3.3	1.6
CARS1	2.6	2.2	5.5	3.3	3.7	6.0	2.0	4.3
CARS2	0.7	1.5	1.1	0.9	1.2	0.9	0.6	1.1
SQRDL	3.0	5.3	3.2	5.6	7.0	0.05	1.0	0.4
ETHE1	2.5	2.6	1.2	2.2	3.5	2.1	2.3	2.2
CDO1	2.6	0.4	4.7	2.1	0.6	2.4	0.4	22.3
CSAD	6.5	2.8	10.1	5.0	5.9	2.3	2.4	7.9
FMO1	2.5	4.8	2.7	7.8	0.3	0.1	0.1	0.1
AHCY	0.4	0.4	0.5	1.1	0.4	0.6	0.8	0.7
AHCYL1	57.0	12.2	14.1	13.8	10.5	15.5	15.3	11.4
AHCYL2	6.8	2.8	1.4	2.6	3.4	7.2	3.5	2.9
DNMT1	2.0	4.0	2.5	2.8	4.2	5.8	2.2	5.3
DNMT3A	8.7	5.7	5.3	10.9	11.8	11.4	14.1	13.4
DNMT3B	0.4	0.5	0.2	0.5	0.6	0.1	0.1	0.2
MAT1A	0	0	0	0	0	0	0	0
MAT2A	53.1	24.7	20.5	26.8	21.2	18.7	34.2	32.8
SLC7A11	27.4	0.9	0.8	123.0	1.3	0.3	0.8	0.4
SLC3A2	35.4	67.0	5.8	51.0	26.5	6.0	8.6	9.2
SLC7A5	4.3	52.5	0.4	1.8	2.7	2.0	0.8	1.7
OTUB1	8.4	6.1	6.8	8.5	9.2	18.0	6.9	9.0
SLC1A1	0.7	14.9	0.5	1.3	1.8	7.2	0.8	28.0
ARL6IP5	6.3	20.2	5.4	6.2	6.8	7.9	7.0	8.0
SLC1A2	680.0	26.6	17.8	32.9	41.0	37.6	73.0	54.3
SLC1A3	520.0	8.6	43.1	72.5	23.9	4.7	21.8	16.5
SLC1A4	19.2	0.5	10.1	1.4	1.8	3.2	0.8	3.9
TXNDC12	5.8	6.8	5.1	4.3	4.9	2.4	4.9	8.2
TMEM161B	4.7	2.1	2.7	2.3	2.3	4.1	1.6	3.2
LMF1	3.8	1.7	2.0	2.7	3.1	1.2	4.8	6.4
ERO1A	1.0	2.4	1.3	1.8	1.1	2.8	1.8	1.7
DNAJC10	4.4	20.8	14.1	12.4	5.2	9.2	4.3	6.8
ERP44	6.6	8.2	5.9	7.7	6.1	3.8	6.2	8.3
TXNDC5	7.4	2.4	10.1	21.7	3.8	4.4	1.0	4.0
TXNDC11	2.4	1.6	1.7	3.6	3.9	3.6	1.0	2.8
TMX4	7.9	6.8	8.5	8.8	12.1	39.5	5.2	12.1
P4HB	12.2	19.3	10.8	23.5	20.9	4.3	16.0	20.0
PDIA3	31.8	47.7	38.1	47.9	66.3	17.2	28.1	61.5
PDIA4	13.3	14.9	55.9	15.6	21.2	5.9	15.0	21.8
PDIA5	0.1	2.3	0.2	1.1	1.3	0.3	0.1	0.8
PDIA6	14.3	18.5	22.4	19.8	25.1	8.4	13.3	27.0

## SUPPLEMENTARY DATA

CHAC1	0.05	0.1	0.1	0.2	0.2	0.6	0.05	0.2
CHAC2	3.2	1.6	1.3	1.3	1.4	2.2	2.2	2.8
LAP3	26.7	8.9	4.6	5.2	12.1	2.0	42.7	18.8
CNDP2	3.8	4.9	3.3	2.8	14.5	3.3	3.3	5.2
DPEP1	0	0	0	0	0	0.02	0	0
ANPEP	0.02	0.2	0.7	7.8	0.3	0.02	0.02	0.3
GGCT	1.2	1.4	2.4	1.1	1.8	3.6	7.3	5.5
OPLAH	8.2	1.0	6.4	2.4	1.1	0.6	0.9	1.7
CDO1	2.6	0.4	4.8	2.2	0.6	2.3	0.3	22.4
CSAD	6.5	2.8	10.3	4.9	5.9	2.5	2.6	8.2
FMO1	2.6	4.7	2.8	7.8	0.3	0.1	0.1	0.1
SLC15A2	16.3	0.5	7.4	0.8	3.6	0.3	1.2	1.3
SLC7A5	4.3	52.5	0.4	1.8	2.7	2.0	0.8	1.7
SLC6A9	27.0	0.4	7.1	4.4	1.0	1.4	13.1	5.7
GGT1	0.5	0.2	0.3	2.3	0.4	0.05	0.3	0.3
GGT5	0.1	0.3	0.2	11.3	3.3	0.05	0.1	0.1
GGT7	1.7	0.3	0.6	0.7	1.8	5.2	1.5	2.7
ABCC1	1.1	0.8	2.4	0.9	1.9	1.4	1.3	2.5
ABCC3	0	0.1	0.2	0.3	7.0	0	0	0.2
ABCC4	0.8	16.7	1.8	1.8	1.1	0.2	0.2	0.9
ABCC5	5.9	2.5	9.9	4.1	5.4	9.3	4.8	5.5
ABCC9	0.2	0.8	0	0.7	0.7	0.1	0.2	0.2
SARDH	3.5	0.8	1.1	1.7	0.5	0.1	0.2	0.5
TXNIP	3.9	20.6	34.5	24.7	41.5	0.2	1.2	2.9
GSTA4	13.9	56.0	33.4	9.2	1.9	3.1	1.1	1.5
GSTM1	131.0	39.0	168.0	58.0	8.8	2.4	15.5	3.7
GSTM2	0.1	4.5	1.4	14.5	0.6	0.02	0.05	0.4
GSTM4	1.0	0.5	2.3	1.0	0.9	0.6	0.6	0.7
GSTM5	64.0	7.2	17.2	10.9	9.2	9.6	38.0	18.4
GSTM6	0.7	0.1	0.9	0.5	0.2	0.3	0.9	0.1
GSTM7	1.4	9.5	3.1	2.7	0.6	0.5	18.2	2.8
GSTT1	4.7	1.0	6.7	11.8	0.6	0.3	0.3	1.6
GSTT2	1.2	5.3	1.0	4.6	0.8	0.1	0.1	0.2
GSTT3	1.7	1.3	0.4	2.1	0.6	0.03	0.2	0.07
GSTK1	10.4	6.0	3.3	7.1	1.7	0.2	5.2	2.8
GSTZ1	6.8	1.3	4.3	2.2	1.3	2.2	3.3	2.7
MGST1	29.5	13.2	28.9	17.6	1.9	0.2	0.7	0.9
MGST3	4.1	10.5	1.9	3.6	5.4	15.5	8.1	5.3
SLC25A39	7.9	7.8	14.7	8.2	6.8	5.0	5.7	7.4
SLC25A40	0.8	1.0	1.4	0.7	0.8	0.7	0.4	0.8
AFG3L2	3.8	3.7	4.4	4.1	3.9	6.8	2.3	3.9