SELENIUM AND SELENOPROTEINS IN BRAIN DEVELOPMENT, FUNCTION, AND DISEASE

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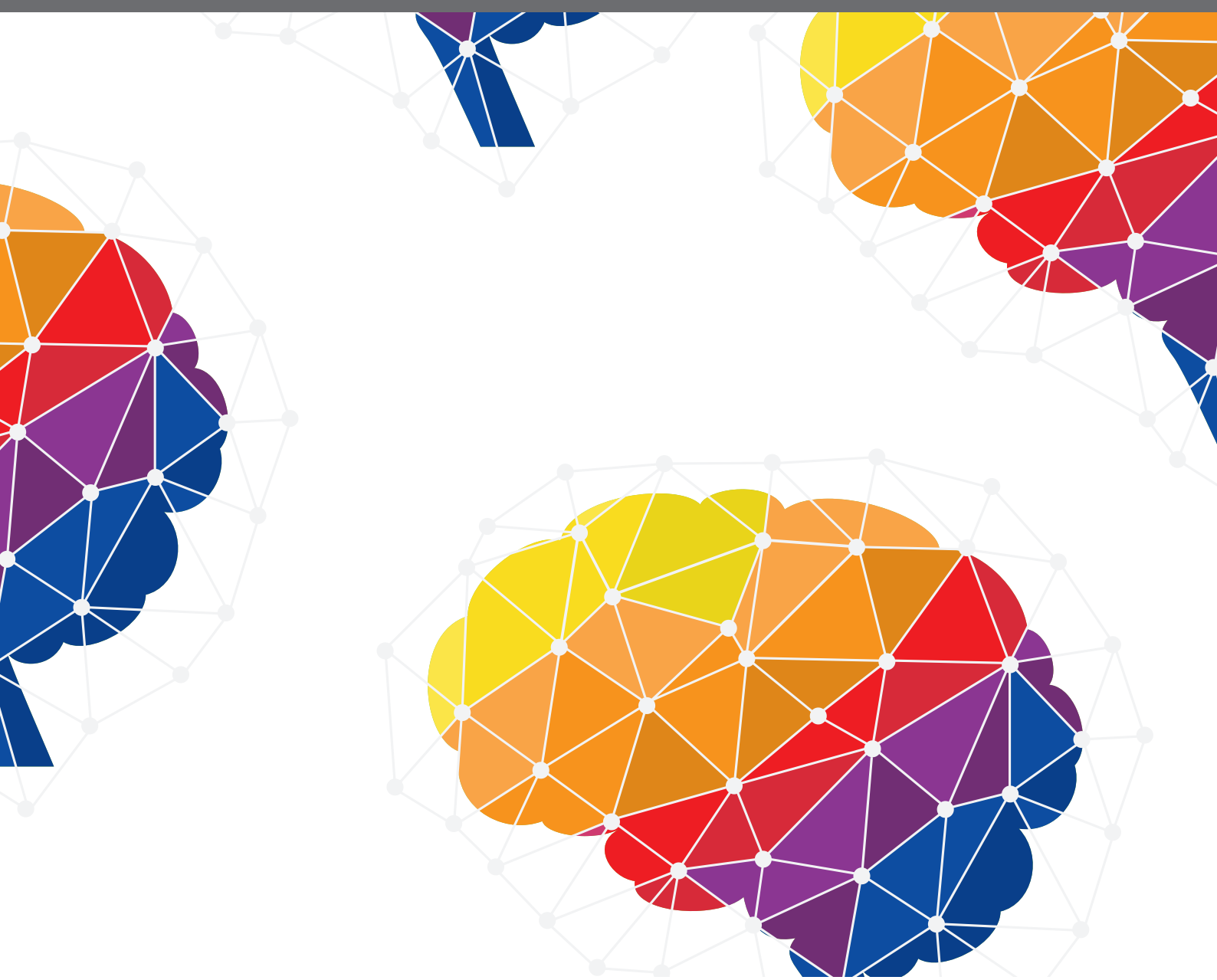

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SELENIUM AND SELENOPROTEINS IN BRAIN DEVELOPMENT, FUNCTION, AND DISEASE

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EDITORIAL
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Editorial: Selenium and Selenoproteins in Brain Development, Function, and Disease

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Keywords: selenium, selenoprotein, brain, neurodevelopment, neurodegeneration, oxidative stress

Editorial on the Research Topic

Selenium and Selenoproteins in Brain Development, Function, and Disease

Selenium (Se) is an essential micronutrient with important effects on the brain and cells of the nervous system. Its influence is mediated primarily through selenoproteins, a class of proteins characterized by the co-translational incorporation of Se as the amino acid selenocysteine. These proteins play fundamental roles in redox signaling, protection from damage, endocrine homeostasis etc. and include the glutathione peroxidases, thioredoxin reductases, and iodothyronine deiodinases. The human genome encodes 25 distinct selenoproteins, along with a host of additional Se-related proteins involved in selenoprotein biosynthesis and Se metabolism. Many of these are highly expressed in brain, and mouse knockout studies have shown that several are indispensable for neurodevelopment and protection from neuronal damage, as e.g., shown for parvalbumin-expressing interneurons, a class of GABAergic neurons characterized by high rates of metabolism. Humans with rare mutations in selenoprotein biosynthesis genes exhibit neurological defects that parallel those detailed in knockout mice, including deficits in cognition and motor function, seizures, hearing loss, and altered thyroid metabolism.

The goal of this Research Topic was to assemble a collection of state-of-the-art articles pertaining to the influence of selenium (Se) and / or selenoproteins on brain development, function, and disease. This resulted in a compilation of four original research and five review articles from Se researchers around the globe.

Several of manuscripts were devoted to the basic science of Se /selenoproteins. Schweizer et al. provide a concise overview of the various roles of individual selenoproteins in brain, along with associated Se-related proteins involved in selenoprotein biosynthesis. These authors also offer food for thought regarding issues that remain unsolved in Se biology. Solovyev et al. present an in-depth review of Se transport and homeostasis at the blood-brain barrier, a matter of great importance to maintenance of proper redox balance in brain. The relationship between stress and selenium homeostasis in brain is explored by Torres, Alfulaij et al. with thorough review of published studies employing selenocompounds in rodent models of stress. Martinez and Hernandez provide newfound insight into the developmental regulation of brain thyroid metabolism, showing that type 3 deiodinase is a critical negative regulator of thyroid hormone action during the fetal period. Also, in an original research report, Kilonzo et al. assess the developmental effects of varying levels of Se supplementation upon neurobehavioral and metabolic indices in adulthood, detailing that Se-deficiency leads to deficits in cognition, altered sensorimotor gating, and increased adiposity. Finally, Torres, Yorgason et al. supply first evidence of a modulatory role for Selenoprotein P in mesolimbic dopaminergic signaling.

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The remaining publications in this collection focus upon the roles of Se / selenoproteins in various disease states. A clinical study conducted by Seelig et al. identify the enigmatic Selenium-Binding Protein 1 as a biomarker for adverse outcomes following traumatic spinal cord injury. Notably, this work builds upon earlier findings of elevated SELENBP1 levels in the context of neuropsychiatric disease (Glatt et al., 2005; Udawela et al., 2015), and the recent demonstration of SELENBP1 as a methanethiol oxidase (Pol et al., 2018). An overview of the potential therapeutic usage of Se in the treatment of glioblastoma is presented by Yakubov et al., with specific focus upon brain edema, glioma-related angiogenesis, and glioma-associated microglia. Lastly, the important topic of Se and Alzheimer's disease (AD) is covered by Zhang and Song. The authors detail links between known functions of individual selenoproteins and pathological alterations in AD, such as elevated endoplasmic reticulum stress, impaired calcium homeostasis, and neuroinflammation.

Collectively, the publications indicate the grown maturity of our knowledge on the essential roles of Se and selenoproteins for brain development and protection from neuronal loss. The wide spectrum of aspects covered by this Research Topic nicely mirrors the expanding understanding of the diverse roles played by the different selenoproteins along with the new perspectives for taking advantage of these insights in the quest for nutritional and therapeutic support in the preservation of our sensory and intellectual functions. We are convinced that the articles published in this Research Topic contribute to this important aim and provide the readership with essential knowledge, stimulating thoughts, and strong motivation along this line.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: LS holds shares in selenOmed GmbH, a company involved in selenium status assessment and supplementation.

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

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REVIEW
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Selenium at the Neural Barriers: A Review

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Selenium (Se) is known to contribute to several vital physiological functions in mammals: antioxidant defense, fertility, thyroid hormone metabolism, and immune response. Growing evidence indicates the crucial role of Se and Se-containing selenoproteins in the brain and brain function. As for the other essential trace elements, dietary Se needs to reach effective concentrations in the central nervous system (CNS) to exert its functions. To do so, Se-species have to cross the blood–brain barrier (BBB) and/or blood–cerebrospinal fluid barrier (BCB) of the choroid plexus. The main interface between the general circulation of the body and the CNS is the BBB. Endothelial cells of brain capillaries forming the so-called tight junctions are the primary anatomic units of the BBB, mainly responsible for barrier function. The current review focuses on Se transport to the brain, primarily including selenoprotein P/low-density lipoprotein receptor-related protein 8 (LRP8, also known as apolipoprotein E receptor-2) dependent pathway, and supplementary transport routes of Se into the brain via low molecular weight Se-species. Additionally, the potential role of Se and selenoproteins in the BBB, BCB, and neurovascular unit (NVU) is discussed. Finally, the perspectives regarding investigating the role of Se and selenoproteins in the gut-brain axis are outlined.

Keywords: selenium, selenoprotein P, low molecular weight selenium species, blood–cerebrospinal fluid barrier, blood–brain barrier, selenium transport, brain-gut axis, LRP8

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INTRODUCTION

The crucial role of the essential trace element selenium (Se) for the brain was already reported in the study of Weber et al. (1991) demonstrating the alleviation of intractable seizures in children with a low level of glutathione peroxidase (GPX) activity following Se supplementation. A commonly accepted Se metabolism concept includes the transformation of dietary Se to hydrogen selenide (HSe^-), which serves as an intermediate between reductive metabolism of Se and excretory pathways, i.e., water-soluble methylated Se compounds (Chatterjee et al., 2003; Ogra and Anan, 2009) and selenosugars (Juresa et al., 2006; Kuehnelt et al., 2006; Rayman et al., 2008). Importantly, hydrogen selenide and its activated form selenophosphate and other reactive low molecular mass (LMM) chemical species of Se are thought to be relevant to the majority of Se biological activity being metabolic precursors of selenoproteins (Loef et al., 2011; Weekley and Harris, 2013). For a detailed description regarding Se absorption and metabolism, the reader is referred to the specialized reviews, e.g., Combs et al. (2013), Roman et al. (2014), Cardoso et al. (2015), and Vindry et al. (2018).

Se is known to contribute to several crucial physiological functions in mammals: antioxidant defense, fertility, thyroid hormone metabolism, and immune response (Hadaszadeh and Beggs, 2006; Rayman, 2012; Schomburg, 2012, 2017; Solovyev et al., 2019). Biological functions of Se in humans (Rayman et al., 2008; Rayman, 2012) manifest themselves primarily via 25 selenoproteins (Savaskan et al., 2003; Arner, 2010; Zhang et al., 2010), highly specialized proteins that have the 21st proteinogenic amino acid selenocysteine (Sec) at their active center (Kryukov et al., 2003; Arner, 2010; Weekley et al., 2011). Hitherto, the functions of a couple of selenoenzymes are rather well described. First of all, antioxidant selenoenzymes are often in the spotlight, including GPXs types I-IV and VI (GPX1-4, 6), thioredoxin reductases type I-III (TXNRD1-3), and methionine sulfoxide reductase B (MrsB) (Arner and Holmgren, 2000; Davis et al., 2012; Rayman, 2012; Brigelius-Flohe and Maiorino, 2013; Kim, 2013). Another relatively well-studied group of selenoprotein are iodothyronine deiodinases type I-III (DIO1-3), which are involved in thyroid hormone metabolism (Köhrle et al., 2000). Other selenoproteins are somewhat less studied, yet their functions seem quite diverse (Papp et al., 2007). For instance, selenoproteins S (SELENOS), N (SELENON), M (SELENOM), T (SELENOT), and F (SELENOF, previously known as 15 kDa selenoprotein (Gladyshev et al., 2016) are endoplasmic reticulum-associated proteins, involved in the unfolded protein response (Bar-Nun, 2005; Ye et al., 2005) and, potentially, other less explored functions.

An important feature of Se metabolism and selenoprotein expression is a highly hierarchic structure. This hierarchy refers to the protein species, organs, and body compartments, with the brain ranging atop of all other organs and tissues. Brain and cerebrospinal fluid (CSF) levels of Se are independent on blood Se level (Tondo et al., 2010; Solovyev et al., 2013); so the brain is protected from Se deficiency (Zhang et al., 2008). The other hierarchic aspect is connected with the patterns of expression of certain potentially more essential selenoproteins (Novoselov et al., 2005) in certain tissues, first of all in the brain, to maintain these important selenoproteins at a high level even at Se deficiency. Conversely, the production of other selenoproteins is severely deprived under Se shortage (Savaskan et al., 2007; Reeves and Hoffmann, 2009; Zhang et al., 2019). Such a Se-utilization hierarchy amongst selenoproteins and body compartments is related to the sophisticated regulation of selenoprotein expression (Papp et al., 2007; Kim et al., 2011). Within the selenoprotein transcription hierarchy, Dio1 holds the top position. The main Se transporting protein – SELENOP – is found in an intermediary position on this selenoprotein transcription ranking. The various forms of GPXs show a scattered picture: GPX2 and GPX4 are less affected by Se deficiency than GPX1 and GPX3 (Sunde, 2012).

Recently, the introduction of genomic, autoradiographic, and proteomic techniques (Guo et al., 2018), as well as advances in chemical speciation (Michalke et al., 2018; Sargent et al., 2019), opened new insights in the studies of brain Se biochemistry and neurotoxicology (Schweizer et al., 2004; Solovyev, 2015). For further details concerning the role of Se in human health (Köhrle et al., 2000; Rayman, 2012; Steinbrenner and Brigelius-Flohé, 2015), the brain and brain disease (Schweizer et al., 2004; Pillai et al., 2014; Cardoso et al., 2015; Solovyev, 2015; Solovyev et al., 2018), metabolism (Steinbrenner and Sies, 2013; Weekley and Harris, 2013; Vinceti et al., 2016), and nutrition (Navarro-Alarcon and Cabrera-Vique, 2008; Torres-Vega et al., 2012) the reader is referred to the specialized reviews.

Se is an essential trace element for the human body and specifically for the human brain (Ingold et al., 2018), but it also can be highly neurotoxic depending on intake and speciation (Rayman, 2012; Vinceti et al., 2014; Solovyev, 2015; Michalke et al., 2018). The nutritional requirement for Se was first demonstrated in 1957 (Schwarz and Foltz, 1957), which was underpinned by the discovery of Se-dependent GPXs (Rotruck et al., 1973). Nevertheless, the optimal dietary intake of Se induced intensive debates for a long time, which are still going on (Sunde, 2006; Vinceti et al., 2013a, 2017b; Roman et al., 2014). Currently, the values of ca. 20–70 (Gammelgaard et al., 2008; Schomburg, 2012; Vinceti et al., 2013a; Weekley and Harris, 2013) or 40–50 μg Se per day are most commonly cited in the literature as an optimum Se intake (Sunde, 2006; Combs et al., 2013). Tolerable upper intake level was set by the Institute of Medicine of the National Academy of Sciences of the United States as 400 μg per day for adults (Boyd, 2011). European Food Safety Authority (EFSA) set adequate Se intake as 70 μg/day for adults and 85 μg/day for lactating women (EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), 2014). For the rodents, the generally recommended levels of Se in the chow are ca. 0.04–0.10 μg Se/g diet (Yang et al., 1989; Sunde et al., 2005; Sunde and Raines, 2011), which may correspond to ca. 1–2 μg Se daily in rats. The exact optimal intake of Se in rodents seems to be dependent on exact breed, age, and Se speciation.

Notably, the neuroprotective role of Se compounds is not exhausted with antioxidant effects of Se species, but also appeared to have a role in *de novo* selenoprotein synthesis, regulation of calcium channels, and mitochondrial biogenesis (Uguz and Naziroglu, 2012). Remarkably both Se-deficient and Se-excessive diet in mice lead to an increased level of iron in the hippocampus; however, in the cerebral cortex, only Se-deficient diet led to iron accumulation (Sharma et al., 2019). Increased iron in brain tissue causes reactive oxygen species (ROS) formation via Fenton reaction, inducing ferroptosis and finally leading to neurodegeneration (Kim et al., 2015; Stockwell et al., 2017; Cobley et al., 2018). This indicates that Se metabolism may cross-effects the regulation of other metal levels and can lead to a wide range of consequences with pathological effects.

Importantly, as for any other nutritional compounds, dietary Se needs to reach effective concentrations in the CNS to exert its vital function (Campos-Bedolla et al., 2014). To do so, Se-species have to cross the blood–brain barrier (BBB) and/or blood–cerebrospinal fluid (CSF) barrier (BCB). Crossing the barriers as well as subsequent promoting antioxidant activity appear to be, to some degree, dependent on the chemical form, since the organic form of Se was proven to be more powerful in increasing the expression and activity of TXNRD, GPX1 and GPX4 (Song et al., 2014). TXNRD plays an important role in maintaining the redox balance and has protective role inside dopaminergic

cells, which are prone to oxidative stress, e.g., under parkinsonian degeneration (Lopert et al., 2012).

Blood–brain barrier and BCB are "guarding systems" of the brain formed mainly by endothelial cells, which separate the central nervous system (CNS) from the general circulatory system of the body, protecting the brain from toxic metabolites and pathogens (Zenaro et al., 2017). BBB and BCB provide trophic support, absorbing nutrients such as amino acids, polyunsaturated fatty acids, and essential trace elements that are vital for brain function (de Wilde et al., 2017). The main interface between the general circulation of the body and the CNS-compartment is the BBB. Endothelial cells of brain capillaries are the primary anatomic units of the BBB, mainly responsible for the barrier function (Abbott et al., 2010). However, brain endothelial cells actively interacting with other brain cells, including neurons, astrocytes, myocytes, pericytes, and extracellular matrix components (Muoio et al., 2014). All these cell types, including BBB endothelial cells, are involved in the regulation of blood circulation, including vasodilation and vasoconstriction, together being referred to as neurovascular unit (NVU).

A peculiar fact on neurodegenerative disorders is that they are normally characterized by an increased ROS production (Loef et al., 2011) and the decline of BBB and BCB (Balusu et al., 2016). For instance, animal (Sengillo et al., 2013) and human studies (Halliday et al., 2016; Skillbäck et al., 2017) indicate the vulnerability of the NVU in Alzheimer's disease, the most common neurodegenerative disease (Muoio et al., 2014), and both protective and trophic functions of the neural barrier seem to be impaired (Balusu et al., 2016; Zenaro et al., 2017).

Upon entering the body through diet, Se is mainly taken by the liver (Burk and Hill, 2015) to be distributed to the extrahepatic tissue. For the details on Se absorption and general metabolism in the body, the reader is referred to the specialized publications (Ogra and Anan, 2009; Burk and Hill, 2015; Shini et al., 2015; Solovyev et al., 2018; Ha et al., 2019). The current review focuses on Se transport to the brain, including, first of all, selenoprotein P/low-density lipoprotein receptor-related protein 8 (LRP8, also known as apolipoprotein E receptor-2) dependent pathway, and supplementary transport routes of Se into the brain via low molecular weight Se-species. Additionally, a potential role of Se and selenoproteins in the BBB, BCB, and NVU is discussed. Finally, the perspectives regarding investigating the role of Se and selenoproteins in the gut-brain axis are outlined.

BLOOD–BRAIN BARRIER, BLOOD–CEREBROSPINAL FLUID BARRIER, AND NEUROVASCULAR UNIT

The mammalian brain is separated from the general circulation system by the BBB, which is localized in the brain capillaries and pia-subarachnoid membranes, and the BCB localized in the *choroid plexus* of the brain ventricles. The primary contribution to the barrier function belongs to the BBB since at the level of brain micro-vessel endothelium BBB is the major site of blood-CNS exchange (Abbott et al., 2010). BBB plays a crucial role in the maintenance of CNS homeostasis (Erickson and Banks, 2013). The functions of the BBB and BCB include: protection of the brain from pathogens and toxic metabolites, the separation of the brain and periphery neurotransmitter pools, intake of essential nutrients and discharge of metabolites, and maintaining the immune privilege of the brain, where the immune activity is mainly accomplished by internal microglia rather than, e.g., bone marrow or thymus-derived immune cells (Galea et al., 2007; Abbott et al., 2010; de Wilde et al., 2017; Zenaro et al., 2017).

These barriers are physically represented by the so-called tight junctions between brain endothelial cells and epithelial cells, attributed to the special proteins such as occludin, claudins, and the associated proteins zona occludens (ZO-1, ZO-2, and ZO-3), which are highly expressed in brain endothelium (Chen et al., 2009; Steinemann et al., 2016). Another aspect of barrier function is related to the functioning of multiple active transporters, which carry nutrients and metabolites in both directions (Campos-Bedolla et al., 2014; Blanchette and Daneman, 2015). Tight junctions produce high transendothelial cell electrical resistance, impeding ions and small charged molecules from crossing the BBB (Blanchette and Daneman, 2015). Tight junctions also support transporter function by limiting lateral diffusion of membrane proteins (Abbott et al., 2010).

A second interface between the CNS and periphery, formed by the epithelial cells of the *choroid plexus* facing the CSF, the CSF *per se*, and the highly permeable ependyma in the brain ventricles, constitute the BCB (Abbott et al., 2010; Spector et al., 2015). The choroid epithelial interface of the BCB acts together with the BBB, maintaining neuron wellbeing (Johanson et al., 2011). The CSF is an excretion of the *choroid plexus* into the brain ventricular system (Brown et al., 2004) and it is in permanent close contact with the brain in the extraparenchymal cave (Aguilar et al., 1998). The blood comes close to the CSF in two main areas of the brain: over the subarachnoid space in the arachnoid membrane blanket and in the *choroid plexus* of the brain ventricles (Johanson et al., 2011). CSF is bathing and sheathing the brain, protecting it from mechanical stress and contributing to brain homeostasis through constant exchange with brain interstitial fluid (Abbott et al., 2010). This fact predestines CSF to be *that* sample type from living subjects to analyze CNS-related exposure, transport efficiency across neural barriers or metabolic changes in the brain due to neurodegenerative conditions (Solovyev et al., 2013). This holds true as well for Se and selenoproteins or other Se-species.

Barrier functions develop prenatally and are well-formed by birth (Goasdoué et al., 2017). Endothelial progenitor cells invade the neural tissue from the surrounding perineural vascular plexus and enter into the neuroepithelium; neural progenitor cells generate molecular signals driving the migration of the endothelial cells, which in turn secrete cues to recruit pericytes; for details see a review by Blanchette and Daneman (2015). Neural barriers are a highly dynamic system, responding to different signals, including local changes and requirements, and able to be regulated via a number of mechanisms and cell types, in both physiological and pathological conditions (Abbott et al., 2010).

Blood–brain barrier and BCB are sophisticated systems for a direct study in a living organism. Therefore, active attempts are being undertaken to design *in vitro* models of these systems. Such

artificial systems could facilitate the investigation of processes across the BBB and BCB. As such models are designed to reproduce and predict the processes across the real barriers. The reliable models must correspond to a relevant set of parameters in the real brain. However, there is still a lack of *in vivo* understanding of many processes at neural barriers, making robust validation of model systems to be associated with noticeable difficulties.

The developed models can be divided into several main types: transwell systems (Helms et al., 2016; Stone et al., 2019), cell aggregate-based models (Urich et al., 2013; Cho et al., 2017), and dynamic systems (Campisi et al., 2018; Jeong et al., 2018; Ahn et al., 2020). In the simplest version, transwell models represent endothelial cells cultured on a matrix-coated permeable membrane inserts for the standard cell culture plates, which divide the cultivation well into two parts, imitating the blood-facing and brain-facing compartments of the barrier. Additionally, astrocytes, pericytes, and neurons can be co-cultured together with endothelial cells to mimic the real vascular environment in the brain more closely (Stone et al., 2019). The advantages of such systems are the simplicity of implementation, low costs, and the possibility to assess the transendothelial electrical resistance (TEER) rather easily as a parameter characterizing modeled barrier integrity. Additionally, such systems are well suited for the screening of permeability coefficients (Wolff et al., 2015); predominantly, in the case of compounds with a passive diffusion mechanism (Garberg et al., 2005). The same transwell membranes can be applied for the modeling BCB (Schroten et al., 2012; Drobyshev et al., 2021). Nevertheless, there is a lack of the relevant cell lines to model the whole sophisticated cell interaction for both BBB and BCB, which is especially problematic for the latter since it is combined with a more model-challenging barrier geometry (Strazielle and Ghersi-Egea, 2011). Overall, non-presentation of some cell types in such models, the absence of blood flow, and a lack of metabolic and neurochemical coupling between the neuronal cells and the barrier components limit the implication of these models (Bagchi et al., 2019).

Dynamic BBB models were designed to overcome the disadvantages of the transwell models associated with the lack of shear stress and close contact of endothelial cells with neuroglia. In these models, endothelial cells and astrocytes are cultured on the inner and outer surface of the porous hollow fibers (He et al., 2014). The culture medium is circulated through the system to achieve shear stress equivalent to that in the physiological conditions. Also, a gas-permeable tubing system is used to keep the O_2/CO_2 balance. However, the dynamic BBB model has a lot of shortcomings: it is not possible to visualize the endothelial cells; these models require much higher cell numbers to build-up a tight monolayer and longer cultivation times to reach stable TEER values (Cucullo et al., 2002, 2011). Nevertheless, as these models allow controlling the medium flow, dynamic BBB models were successfully applied for the investigation of the ischemia-induced injury (Cucullo et al., 2008) and antiepileptic drugs (Cucullo et al., 2007). The introduction of microfluidic devices was the next step in the development of dynamic BBB models (Wolff et al., 2015). Due to the miniaturization of the flow chambers and the limitations of the membranes, the conventional dynamic BBB models were mostly discontinued. At the same time, the small size of the flow chambers limits their application for modeling shear stress. However, the active development of the microfluidic BBB systems in recent years demonstrates the potential of these models for a variety of research tasks (Adriani et al., 2017; Jeong et al., 2018; Bhalerao et al., 2020).

Cell aggregate models or "spheroid" models consist of endothelial cells, astrocytes, and pericytes, which are able to self-organize into spherical structures with astrocyte core, surrounded by pericytes and covered with endothelial cells (Urich et al., 2013). Such systems may become a viable alternative to the transwell or microfluidic models for certain implications. The main advantage of these systems is a direct contact between the barrier cells (Gastfriend et al., 2018). Accordingly, the disadvantage of these models is the absence of a simple way to assess barrier function such as TEER measurement and complicated permeability screening (Cho et al., 2017). At the moment, such systems seem to be the most suitable for studying the effects of various compounds on the constitutional cells of the barrier (Nzou et al., 2018; Leite et al., 2019), rather than directly on the barrier functions.

There is a large set of requirements for barrier models: strong barrier function, the presence of a wide range of transporters and receptors, regulation of immune cell trafficking, mimicking a complex interaction of several types of cells, as well as, a dynamic balance between the cells. That makes the implementation of the *in vitro* BBB or BCB models extremely difficult. However, a deeper understanding of the complex nature of BBB and BCB together with the development of the new models and the improvement of the current barrier-modeling techniques indicates that they may become a very useful research tool for studying BBB and BCB in the future. This may include the research on the nutrient transport to the brain tissue and barrier dynamics, including modeling of the NVU functionality.

The concept of NVU was introduced as a structure formed by neurons, astrocytes, basal lamina covered with smooth muscle cells and pericytes, endothelial cells (components of the BBB), and extracellular matrix (Harder et al., 2002). This cellular complex detects the neuronal supply and triggers necessary responses, vasodilation or vasoconstriction, via their anatomical and chemical relationship (Muoio et al., 2014). Importantly, brain endothelial cells are known to gain their specialized BBB functions through interactions with other cells of NVU such as pericytes, astrocytes, and neurons (Canfield et al., 2019), which is crucial for the development, regulation, maintenance of the neural barriers (Daneman et al., 2010a,b).

The decline of BBB and BCB are involved in many neurological diseases (Blanchette and Daneman, 2015), including, e.g., Alzheimer's (Erickson and Banks, 2013; Zenaro et al., 2017) and Parkinson's disease (Gray and Woulfe, 2015), epilepsy (Oby and Janigro, 2006), etc. In this respect, BBB is currently drawing more interest if compared to BCB. To conclude, neural barriers, first of all, BBB and other aspects of NVU is a dynamically developing branch of brain research and they may be expected to gain recognition as valid therapeutic targets in the future (Campos-Bedolla et al., 2014).

SELENIUM TRANSPORT TO THE BRAIN – SELENOPROTEIN P AND LOW MOLECULAR WEIGHT SELENIUM-SPECIES

Se is an essential trace element necessary for adequate brain function (Cardoso et al., 2015; Solovyev, 2015); however, its uptake by the neuronal tissue should be strictly regulated to prevent toxicity (Burk and Hill, 2009, 2015). Currently, the role of disturbed trace element homeostasis and metal exposure in the brain is being studied intensively. The loss of barrier integrity promotes increased brain exposure to circulating metabolites, inorganic ions, and circulation proteins, which in healthy conditions either cannot enter the brain completely or in a strictly controlled manner only. Both metal ions and leaked proteins (Linert and Kozlowski, 2012; Choi et al., 2017) may modulate amyloidgenesis and other pathological processes in the brain after a "ticketless" transfer through the barrier. Increased brain exposure to mineral elements, present as low-molecular-weight species, bypassing the deteriorating neural barrier may contribute to the general pathologic processes in the brain.

The initial understanding of brain Se transport came from the use of ^{75}Se-radioactive tracer experiments (Burk et al., 1991, 2003; Hoppe et al., 2008; Kuhbacher et al., 2009). The presence of ^{75}Se in the brain after the injection of labeled ^{75}Se-selenite to Se deficient rats was observed only after the appearance of ^{75}Se-Selenop in the blood plasma, differentiating the brain from other tissues (Burk et al., 2003). Furthermore, the injection of ^{75}Se-labeled Selenop caused five-time higher accumulation of ^{75}Se in the Se-depleted rat brain 2 h later than that in Se-sufficient animals (Burk et al., 1991). For more details regarding the early studies on body Se transport, the reader is referred to the review by Chen and Berry (2003).

In recent years, our understanding of Se transport to the brain improved considerably. The central role in Se transport is attributed to SELENOP, a sole selenoprotein in mammals and other vertebrates, containing multiple Se atoms as Sec residues (Kryukov et al., 2003). The biosynthesis of SELENOP, involving the incorporation of multiple Sec moieties, is modulated by two SECIS elements in the 3′ UTR region of SELENOP mRNA, reviewed by Shetty and Copeland (2018a). High high-energy demand from the cell for the incorporation of 7-17 or more, up to 35 (Shetty and Copeland, 2018b), Sec residues, depending on the biological species (Labunskyy et al., 2014), indicates the importance of the protein for the body. SELENOP contains two histidine-rich stretches in the N-terminal domain, which may bind to heparin (Hondal et al., 2001). This differs from the majority of heparin-binding proteins, which bind through basic amino acid sequences containing primarily lysine and arginine (Hileman et al., 1998), but is common for histidine-proline-rich glycoprotein (HPRG) (Burch et al., 1987). Additionally, SELENOP contains a separate heparin-binding site in the N-terminal domain (Burk and Hill, 2009, 2015). Recent studies also showed possible detoxification role of SELENOP, which results from the binding affinity for transition metals such as mercury (Liu et al., 2018). Although SELENOP seems to be a multifunctional protein (Schweizer et al., 2016; Brigelius-Flohe and Flohe, 2017; Solovyev, 2020), body Se transport seems to be its most crucial role (Lobanov et al., 2009). SELENOP is a secreted heparin-binding glycoprotein (Yang et al., 2000), containing ten Se atoms in humans (Chen and Berry, 2003; Rayman, 2012). Circulating SELENOP is mainly produced by the liver (Burk and Hill, 2009; Pillai et al., 2014; Short et al., 2018); however, intracellular expression of SELENOP was reported for neurons (Scharpf et al., 2007), astrocytes (Yang et al., 2000; Steinbrenner et al., 2006), testicular Leydig cells (Koga et al., 1998), adipocytes (Zhang and Chen, 2011), and β-cells of the pancreas (Steinbrenner et al., 2013), at least *in vitro*. Full-length SELENOP and shorter truncated isoforms are detected in the circulation, the latter corresponding both to termination of SELENOP translation at one of the Sec UGA codons (Ma et al., 2002; Kurokawa et al., 2014a) and the action of the proteases (Saito et al., 2004; Kurokawa et al., 2014a,b). SELENOP considerably contributes to the maintenance of body Se homeostasis, mainly orchestrated by the liver (Steinbrenner and Sies, 2009; Solovyev, 2020). The liver directs Se toward essential selenoproteins biosynthesis or excretion (Papp et al., 2007).

As was already mentioned, the human body maintains a specific Se hierarchy (Steinbrenner and Brigelius-Flohé, 2015). The brain ranks high in this hierarchy, being able to maintain relatively high selenoprotein expression under Se deficiency (Burk and Hill, 2009; Solovyev, 2015; Solovyev et al., 2018); together with regulation of selenoprotein expression, SELENOP-dependent Se uptake to the brain seems to play an important role in maintaining this strict hierarchy. In the brain, SELENOP is primarily expressed in astrocytes, but neurons have also been identified as a source of endogenous SELENOP through the entire brain (Steinbrenner et al., 2006; Scharpf et al., 2007), with particularly elevated expression in the putamen and *substantia nigra* (Bellinger et al., 2012). The regulation of SELENOP synthesis seems to be even more sophisticated than that for other selenoproteins, due to the necessity to incorporate multiple Sec elements (Shetty and Copeland, 2018b). As a Se transport protein, SELENOP significantly contributes to Se-dependent brain pathways, including: redox signaling, protein folding, neurochemical signal transduction, and cytoskeleton assembly (Loef et al., 2011; Cardoso et al., 2015).

The majority of the extrahepatic tissues depend on receptor-mediated uptake of SELENOP to maintain adequate selenoprotein expression. **Figure 1** illustrates body Se homeostasis and Se transport, based on several sources (Burk and Hill, 2009, 2015; Ogra and Anan, 2009; Solovyev et al., 2013). First of all, the brain, testes, placenta, and kidney rely on receptor-mediated endocytosis of SELENOP. Se delivery to neurons by SELENOP is accomplished via its receptor, low-density lipoprotein receptor-related protein 8 (LRP8, also known as ApoER2, **Figure 1**) (Burk et al., 2007). SELENOP enters the brain from blood plasma by docking with LRP8 at the BBB in brain capillary endothelial cells (BCECs) and *choroid plexus* epithelial cells (Burk et al., 2014). In other body compartments, LRP8, or another membrane receptor – megalin (also known as LRP2) – is used for SELENOP uptake

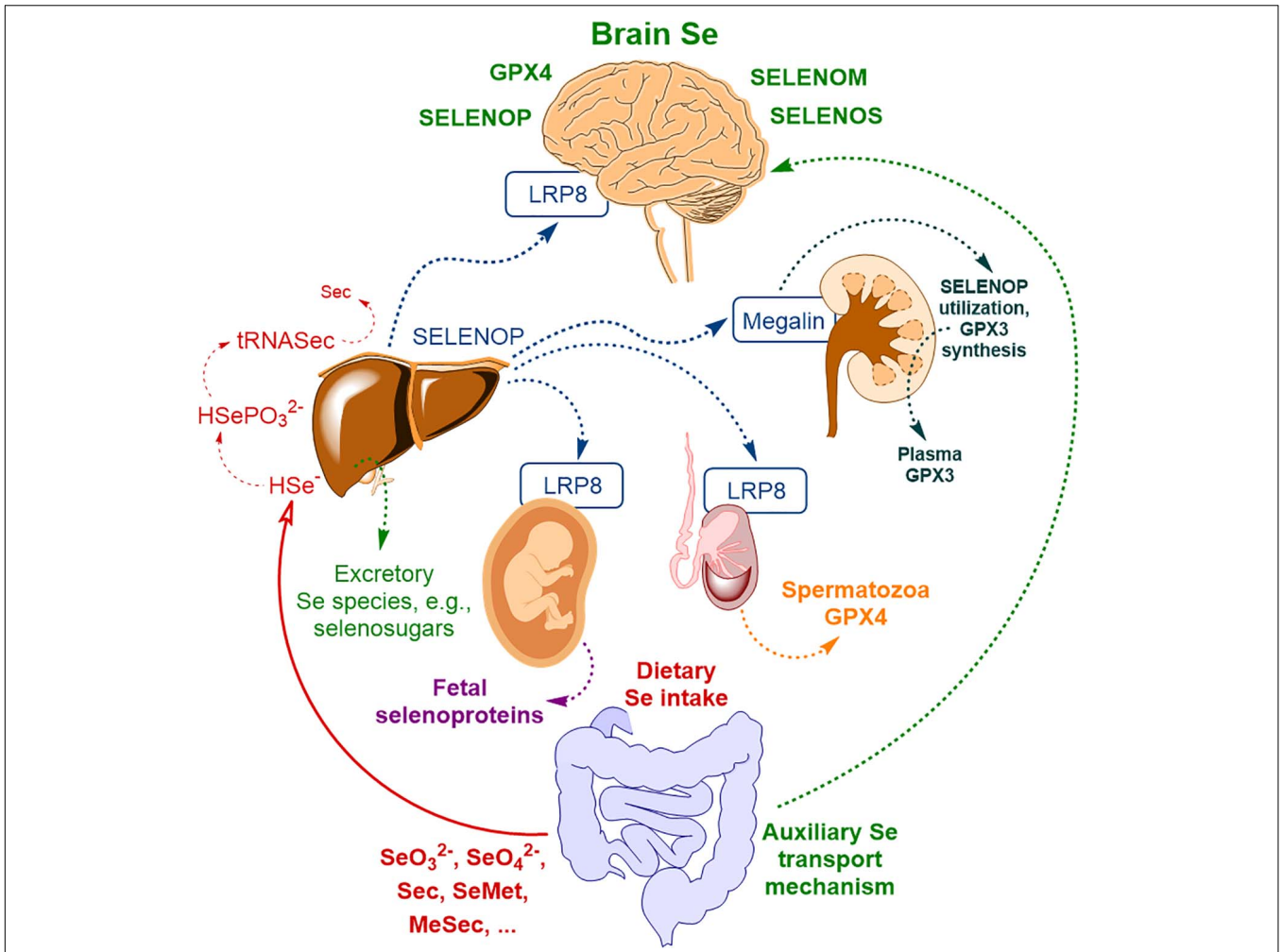

FIGURE 1 | The scheme of body Se homeostasis. Abbreviations: LRP8 – low-density lipoprotein receptor-related protein 8 (LRP8, also known as apolipoprotein E receptor-2, ApoER2), GPX3 – glutathione peroxidase type III, GPX4 – glutathione peroxidase type IV, Sec – selenocysteine, MeSec – methyl selenocysteine, SELENOM – selenoprotein M, SELENOP – selenoprotein P, SELENOS – selenoprotein S; * – auxiliary brain Se transport mechanism, independent of SELENOP, possibly related to selenosugars (Burk and Hill, 2015) and other low molecular weight Se-species (Solovyev et al., 2013) and possibly other minor contributors (please, see text for more detail). Based on Solovyev et al. (2018) with modification.

(Olson et al., 2007, 2008; Chiu-Ugalde et al., 2010; Kurokawa et al., 2012, 2014b); SELENOP *per se* was reported to be in oxidized form for the uptake to take place (Shetty et al., 2018).

This primary mechanism of brain Se uptake was postulated based on mice transgenic studies (first of all, using *Selenop*$^{-/-}$ mice) and is relatively well explored by now. Genetic ablation of SELENOP or LRP8 results in diminished brain Se levels (Hill et al., 2003; Schomburg et al., 2003; Burk and Hill, 2009; Burk et al., 2014) and severe neurological dysfunction upon administration of a Se-deficient diet (Hill et al., 2004; Valentine et al., 2008). The study of *m*RNA levels of selenoprotein and selenoproteom-related genes in *Selenop*$^{-/-}$ mice indicated a considerable reduction of brain selenoprotein expression compared to wild-type mice (Hoffmann et al., 2007). Specifically, the selenoproteins with relatively high expressions in the brain – *Gpx4*, *Selenom*, and *Selenok* – were significantly affected, whereas for selenoprotein W [*Selenow*, an antioxidant selenoprotein with not yet fully understood functions (Whanger, 2009; Yao et al., 2013, 2016)] the expression became nearly undetectable (Hoffmann et al., 2007). Another SELENOP uptake receptor, megalin may also contribute to Se transport to the brain. Megalin is mainly responsible for Se uptake by the kidney (**Figure 1**) and prevents the discharge of SELENOP in the urine (Olson et al., 2008; Kurokawa et al., 2014a,b). Megalin was demonstrated to be present in the *choroid plexus* of the BCB (Carro et al., 2005; Dietrich et al., 2008); however, its exact contribution to brain Se transport was not systematically studied and it seems to be rather limited since *megalin*$^{-/-}$ mice do not exhibit neurological phenotype associated with Se deficiency (Kurokawa et al., 2014b), typical for *Selenop*$^{-/-}$ or *Lrp8*$^{-/-}$ mice.

In a recent study, Sasuclark et al. (2019) explored the cell-type-specific expression of Se-related genes in the mouse and human brain using single-cell RNA sequencing. Transcriptomic data was analyzed in 23,822 mouse and 15,928 human cells for

the genes of 22 selenoproteins and 12 other genes, associated with Se-transport and/or metabolism. Different cell types were investigated. High level of expression of LRP8 was observed for brain endothelial cells of the BBB. SELENOP expression considerably overlapped with that in glial fibrillary acidic protein-positive astrocytes and was generally more prominent in white matter. Additionally, SELENOP expression was most robust in the *choroid plexus* and regions lining the brain ventricles (Sasuclark et al., 2019), which is in line with the previous studies (Rueli et al., 2015). Generally, in accordance with the previous findings (Zhang et al., 2008), Sasuclark et al. (2019) indicated that DIO2, SELENOP, and Se-binding protein 1 (SELENBP1) were predominantly expressed in non-neuronal cells (interestingly, SELENOP was co-expressing with SELENBP1 in astrocytes), whereas the vast majority of selenoproteins and Se-related proteins were most abundant in neurons. Importantly, SELENOP expression was maximal in adjacent astrocytes, rather than the ependymal cells directly lining the ventricles. The authors proposed the following model of Se uptake to the brain via SELENOP-LRP8: SELENOP present in blood and CSF is taken up by LRP8-positive cells of BBB and BCB, resynthesized in neighboring astrocytes, and subsequently released to supply LRP8-positive neurons within the brain with Se – **Figure 2**. SELENOP is known to cross the BCB, being the most abundant selenoprotein and Se-species in the CSF (Solovyev et al., 2013; Mandrioli et al., 2017). For instance, for a collective of 24 neurologically healthy sample donors, a good, age-consistent BBB-integrity value of albumin quotient (Q_{HSA}) of 5.25×10^3, but higher Q-values for GPX ($Q_{GPX} = 8.31 \times 10^3$) and TXNRD ($Q_{TXNRD} = 21.34 \times 10^3$) were observed, demonstrating active transport into the brain-CSF compartment (Solovyev et al., 2013). For SELENOP, even higher CSF-blood quotient ($Q_{SELENOP} = 91.24 \times 10^3$) was reported (Michalke et al., 2017). It may indicate the increased SELENOP transport across the neural barriers, due to high expression of LRP8 at the BBB, which keeps Se levels relatively stable, even during deficiency periods making

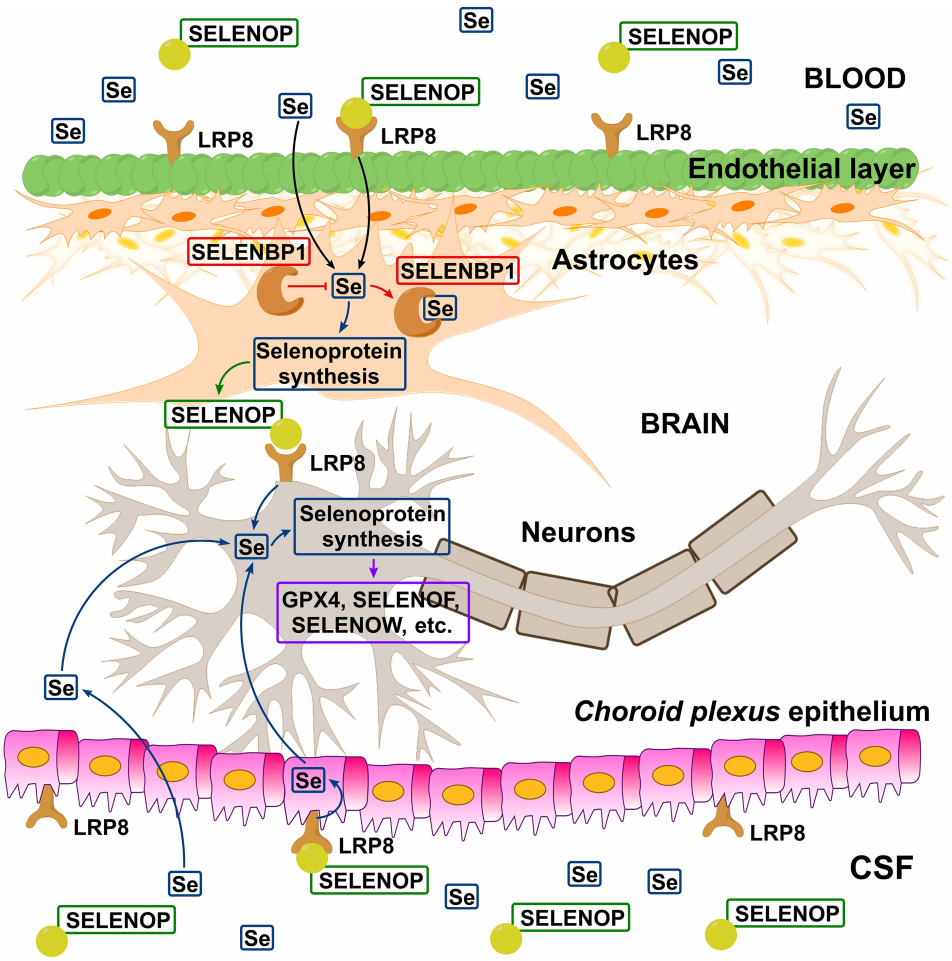

FIGURE 2 | Hypothetical model of Se transport across blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier (BCB). Circulating SELENOP present in blood and CSF is taken up by LRP8-positive cells in the epithelial (BBB) and ependymal (BCB) layers, resynthesized in neighboring astrocytes, and released to supply LRP8-positive neurons with Se. In the astrocytes, SELENBP1 sequesters Se from selenoprotein synthesis and thus negatively regulating SELENOP production. There is also evidence indicating the existence of the SELENOP-independent Se uptake pathway (**Figure 1**). Reproduced from Sasuclark et al. (2019) with modification.

an adequate Se pool available in the CNS (Zachara et al., 2001). The above-proposed hypothesis is quite intriguing and certainly requires further insight.

Recent investigations by Jin et al. (2020) have shown, that human hepatocellular carcinoma (HepG2) cells secrete SELENOP mainly within exosomes, which are stable against cleavage by plasma kallikrein protease (Saito et al., 2004). Additionally, *in vitro* experiments showed that exosomal SELENOP potentially crossed the BBB and supplemented Se to neuronal cells (mouse neuroblastoma N2a cells), inducing the production of intracellular selenoproteins. Exosomes are a subclass of extracellular vesicles of endosomal origin, which are released from the cells for extracellular communication by the transportation of proteins, DNA or RNA (György et al., 2011). The size of exosomes is ∼40–100 nm in diameter and they are enveloped with a lipid double layer as an outer membrane. The function of exosomes in cell-to-cell communication, protein or RNA transport, immune response regulation, antigen presentation, and non-classical secretion of proteins is reviewed by Simpson et al. (2009). Additionally, Jin et al. (2020) indicated the possible involvement of apolipoprotein E (ApoE) in the regulation of exosomal SELENOP secretion and transport, which probably needs further *in vivo* confirmation. For more detailed information about the cellular uptake of exosomes, the reader is referred to the review of Mathieu et al. (2019). Although further studies on secreted exosomal SELENOP are required, the exosomal transport of selenoprotein through BBB to neuronal cells might be an alternative route for Se delivery into the brain.

The mechanism of SELENOP intracellular turnover is not fully clear. Lysosomal degradation of SELENOP was reported (Kurokawa et al., 2012; Shetty et al., 2018); however, the exact proteolysis pathway requires further insight. Se liberated from SELENOP must then be recycled for the production of new selenoproteins. Selenocysteine β-lyase (Scly), an enzyme that seems to play an important role in Se metabolism, releasing Se atoms from Sec (Seale et al., 2018a; Seale, 2019). The idea that Scly may be responsible for SELENOP's Se recycling came from the fact that Scly-depleted HeLa cells exhibited a significant decline of selenoprotein production in the case SELENOP was used as a Se source (Kurokawa et al., 2011).

If SELENOP, as a source of Se for selenoprotein synthesis, acts via Scly, it might deliver the highly reactive Sec residues directly to Scly or through an intermediate, in order to decompose Sec and recycle Se (Seale, 2019). It is worth noting that although *Scly*$^{-/-}$ mice have reduced selenoprotein expression, they do not exert any of the *Selenop*$^{-/-}$ phenotypes like male sterility or severe neurologic defects (Raman et al., 2012; Byrns et al., 2014). Byrns et al. (2014) explored the phenotype of double-knockout *Selenop*$^{-/-}$/*Scly*$^{-/-}$ mice, indicating exacerbated neurological phenotype compared to *Selenop*$^{-/-}$ mice, including motor coordination, audiogenic seizures, and brainstem neurodegeneration. *Selenop*$^{-/-}$/*Scly*$^{-/-}$ animals were shown to require supra-physiological Se supplementation to survive (Byrns et al., 2014). Interestingly, the neurological dysfunction related to the inhibition of GABAergic neuron maturation in male double-knockout *Scly*$^{-/-}$/*Selenop*$^{-/-}$ mice could be prevented by prepubescent castration (Pitts et al., 2015). This suggests a competition between the testes and the brain regarding Se-distribution under Se-deficiency or disrupted Se-homeostasis.

The presence of alternative SELENOP-independent transport pathways for Se was identified in the early studies in *Selenop*$^{-/-}$ mice fed Se sufficient diet (Hill et al., 2003; Schomburg et al., 2003). Up-to-now, these pathways are considerably less explored than the main SELENOP/LRP8 pathway, probably, owing to their supplementary function, which may take over only under specific conditions such as SELENOP or LRP8 deficiency. SELENOP-independent Se transport to neuronal tissue may be attributed to selenosugars (Burk and Hill, 2015) and/or other low molecular weight Se-species (Solovyev et al., 2013). Se conversion into methylated species or selenosugars are Se detoxification pathways present in many biological species. However, there is a lack of understanding of the relationship between specific and non-specific Se metabolism (Tobe and Mihara, 2018). Excretory Se-species, such as selenosugars and trimethylselenonium cation ($TMSe^+$), were shown to be non-toxic for the astrocytoma and other cell types, compared to selenite (Marschall et al., 2016). These compounds are rather intensively produced under supra-nutritional Se intake (Itoh and Suzuki, 1997; Suzuki et al., 2005; Tsuji et al., 2009), and may contribute to brain Se transport under SELENOP or LRP8 deficiency. In this respect, selenosugars seem to be more feasible candidates, since they might exploit glucose- or other transporters (Campos-Bedolla et al., 2014) to cross the BBB and/or BCB whereas $TMSe^+$ is unlikely to be either effectively transported to the brain due to its positive charge and missing capability of effective metabolization in the brain tissue (Suzuki et al., 2006; Jackson et al., 2013). Furthermore, $TMSe^+$ seems not to be regularly appearing in human biofluids. Jäger et al. (2016) reported that $TMSe^+$ was present among Se-excretory species only for a small fraction of the population. Finally, GPX3 as a secreted isoform of GPX (Steinbrenner and Sies, 2009; Brigelius-Flohe and Maiorino, 2013) may also contribute to supplementary Se transport to the brain. Blood serum GPX3 (**Figure 1**) is mainly produced by the kidney (Olson et al., 2008) and GPX3 was detected in human CSF (Solovyev et al., 2013; Vinceti et al., 2019), whereas a rather low level of GPX3 expression was reported for the rat *choroid plexus* (Kratzer et al., 2013). Notably, GPX3 contribution to Se transport to the CNS under normal conditions seems to be far lower compared to the SELENOP-associated pathway.

Recently, Seale et al. (2018a) presented *in vivo* results on Scly expression and activity under the absence of Sec-rich Selenop, indicating the presence of other pathways of maintaining Sec supply for Scly, which remain to be identified. Dietary Se-species, first of all, SeMet and selenite may substitute SELENOP deficiency. For instance, enhanced reduction of selenite and accelerated trans-selenation pathway for SeMet [analog of trans-sulfuration mechanism, transferring sulfur from methionine to serine to yield cysteine (Jackson et al., 2013)] may be additional sources of Se for selenophospate synthesis and consequently selenoprotein production (Seale, 2019). The activation of these pathways may accelerate the induction of dietary Se-species into selenoprotein synthesis (Esaki et al., 1981; Kumar et al., 1992).

Selenite can be reduced to selenide and elemental Se by the action of TXNRD1 (Kumar et al., 1992), an essential selenoprotein ranking high in the selenoprotein hierarchy (Kuhbacher et al., 2009). The scheme of Scly role in Se metabolism is demonstrated in **Figure 3** (Seale, 2019). To conclude, the story of Se recycling still poses some unanswered questions; for instance, the exact cellular localization of Scly, whether Scly directly transfers Se to selenophosphate synthetase 2 (SEPH2) or there are other proteins involved, and the exact role of minor intermediates such as selenohomocysteine (trans-selenation pathway) or Se bound to glutathione (selenite reduction pathway) in Se turnover (Seale et al., 2018a; Seale, 2019).

Inorganic Se (selenite and selenate) may cross the BBB and BCB using inorganic anion transporters, which are present in the barriers (Campos-Bedolla et al., 2014). Sulfate transporters [e.g., SLC13: human Na^+-sulfate/carboxylate co-transporter family (Hu et al., 2020)] may be responsible for carrying selenate across the barrier since it is isomorphic to sulfate. The presence of selenate was observed in human CSF (Vinceti et al., 2013b, 2019; Mandrioli et al., 2017; Violi et al., 2020); however, it is usually mainly attributed to the decaying of CSF selenoproteins, first of all, that of SELENOP (Michalke and Berthele, 2011; Solovyev et al., 2013). Selenite may also employ some inorganic ion transporters but this requires a further warrant. The transport of inorganic Se into the brain appears to be mainly responsible for Se neurotoxicity (Vinceti et al., 2014, 2016), implementing the U-shaped effects of Se on human health (Rayman et al., 2018; Seale et al., 2018b). However, such inorganic Se delivery may become beneficial under severe Se deficiency or malfunction of SELENOP/LRP8 delivery system.

In turn, organic dietary species of Se such as selenoamino acids (SeMet, Se-methylselenocysteine, and to the lesser extent Sec) seem to be capable of entering the brain via aminoacid transporters and, possibly, other routes. Notably, the corresponding mechanistic relationships remain to be elucidated. The key enzyme of the trans-selenation pathway (cystathionine γ-lyase) is known to be expressed in the brain (Diwakar and Ravindranath, 2007; Patel et al., 2018; Seale et al., 2018b). Finally, a minor alternative pathway of Se entering the brain may be related to proteins leaking through the BBB (or BCB), first of all, under pathological conditions, impairing the barrier function, but this notion is rather speculative at the moment. Any general

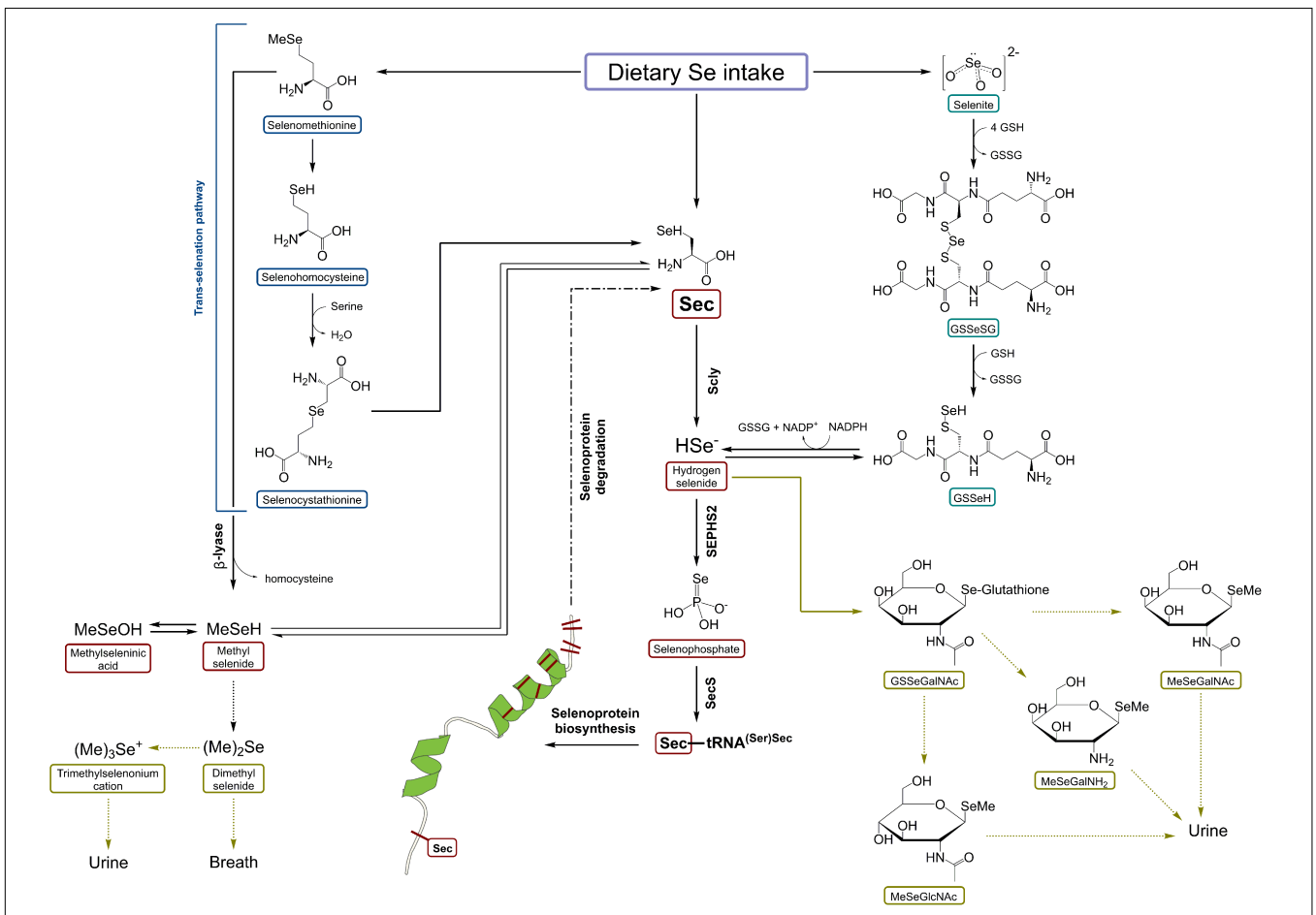

FIGURE 3 | A schematic representation of selenocysteine β-lyase role in Se metabolism. Scly – selenocysteine β-lyase; Sec – selenocysteine; SeMet – selenomethionine; HSe^- – hydrogen selenide; GSSeSG – selenodiglutathione; GSSeH – selenoglutathione; SEPHS2 – selenophosphate synthetase 2. Based on Seale (2019) with modification.

body proteins contain Se as a non-specific substitute for its sulfur analog methionine (Ogra and Anan, 2009). Thus, non-specific leaking of the proteins through the barrier (Pardridge and Mietus, 1980; Lin et al., 2016) may deliver some Se to the brain cells. Particularly, selenized human serum albumin is detected in human CSF (Solovyev et al., 2013; Letsiou et al., 2014). However, the actual contribution of such "backdoor" transport pathways remains elusive.

SELENIUM AND THE GUT-BRAIN AXIS

The human gastrointestinal tract is inhabited by the numerous microorganisms of varied species from different domains of Life, including viruses, archaea, protozoa, bacteria, fungi, and eukaryota (Qin et al., 2010; Welcome, 2019). There is growing evidence of a direct link between gastrointestinal function and the brain (Caracciolo et al., 2014). The gut-brain axis is a bidirectional neurohumoral communication system between the CNS and the enteric nervous system (Collins et al., 2012). For instance, traumatic brain injury activates the gut-brain axis and increases intestinal permeability (Patel et al., 2016); on the other hand, changes of gut microbial composition during neurodevelopment in early life may be detrimental for the CNS and leads to neurological disorders in later life (Louwies et al., 2019). The effect of the gut microbiota on the host's health is related to the production of biologically active compounds *per se*, competing with the host for essential nutrients, and affecting the host's immune system (de Vos and de Vos, 2012; Caracciolo et al., 2014; O'Mahony et al., 2015; Yang et al., 2020) and epigenome (Louwies et al., 2019). Intestinal Se absorption depends on the chemical speciation of the element as well as other factors such as the individual's sex, age, nutritional status, and the composition and activity of the intestinal microbiome (Peters et al., 2018).

The presence of several key selenoproteins including GPXs, SELENOM, SELENOP, and SELENOS as well as SELENBP1 was reported for the intestine, Se status thus affecting gene expression, signaling pathways, and cellular functions in the small and large intestine as well as the gut microbiome composition (Speckmann and Steinbrenner, 2014). Se deficiency is detrimental for the gut barrier function, inducing the disordered intestinal immune response in mice. Additionally, it reduces the levels of neuroactive substances, such as serotonin and melatonin (O'Mahony et al., 2015; Zhai et al., 2019), which are involved in the gut-brain axis (Mawe and Hoffman, 2013; Carabotti et al., 2015). Furthermore, pathological alteration of gastrointestinal flora may lead to diseases, such as inflammatory bowel disease and cancer. The role of Se in these processes remains to be elucidated. The role of Se in the gut disease is outside the scope of the current review and the reader is referred to the specialized publications (Rannem et al., 1998; Speckmann and Steinbrenner, 2014; Kudva et al., 2015; Peters et al., 2018; Kipp, 2020).

The gut microbiota is metabolically highly active and it produces a range of different compounds, including neuroactive molecules, such as acetylcholine, catecholamines, γ-aminobutyric acid, histamine, melatonin, and serotonin. These molecules are essential for regulating peristalsis and sensation in the gut (Petra et al., 2015). Additionally, the presence of gut microbiota considerably affects the uptake and metabolism of the nutrients. Up to 25% of all bacteria have selenoproteins in their genomes (the number varies from 0 to 57) and, thus, they require Se for their growth and metabolism (Kasaikina et al., 2011; Zhang et al., 2019).

In the study of Kipp et al. (2009), male mice were kept on diets for 6 weeks, simulating Se-sufficient (150 μg/kg Se as SeMet) and moderately Se-deficient (86 μg/kg Se) diets in humans. Even this narrow decrease in Se net intake caused the alteration of 952 genes expression – 772 genes were down-regulated and 230 genes were found to be up-regulated. The following pathways were shown to be affected: regulation of protein biosynthesis, response to stress, inflammation, carcinogenesis, and the Wnt pathway (Kipp et al., 2009). In a later study, Kasaikina et al. (2011) studied the composition of gut microbiota in mice kept on Se-deficient, Se-sufficient, and Se-excessive diets. High-throughput sequencing was used for the purpose. They showed gut microbiota to be able to partially sequester dietary Se, limiting its uptake by the host. The authors also pointed out that dietary Se affected both the composition of existing microbiota and the establishment of gastrointestinal microflora (Kasaikina et al., 2011). In the recent experiments in rats, it was shown that high doses of Se (as selenite) partially restored the ranks of phylum and genus of the gut flora after the exposure to methylmercury. The authors also pointed out that the host's Se level was related to the state of the gut microbiome (Liu et al., 2019). Gangadoo et al. (2019) reported that exposure to Se nanoparticles affected the diversity and structure of chicken caecal microbiota *in vitro*. To conclude, it is tempting to speculate that the alterations of the host organism due to Se dietary levels (Kipp et al., 2009, 2012; Peters et al., 2018; Zhai et al., 2019) may be partially related to the gut microbiota. However, further studies should support such a hypothesis.

Another important aspect of the gut microbiota in line with the scope of the current review is related to its role in maintaining BBB integrity. Pathological alterations in gut microbiota induce the increased production of toxic metabolites and reduced production of beneficial compounds like short-chain fatty acids. The metabolic change affects the balance of pro-inflammatory and anti-inflammatory cytokines and other immune factors, promoting the decline of the gut epithelial barrier. This results in concomitant activation of local and distant immune cells and dysregulation of enteric neurons and glia (Welcome, 2019). Gut flora also appears to have a role in the induction of BBB properties; in the absence of normal gut microbiota in the mouse dams, the expression of BEC claudin-5 and occludin is diminished and an increase in BBB permeability is observed in the offspring (Braniste et al., 2014). Notably, more research is currently required to shed light on the exact molecular switches that control the processes in the histohematic barriers of the gut and brain (Welcome, 2019). Even less information exists regarding the role of Se in these processes. There was a report that Se uptake (as selenite) to the brain increased in lipopolysaccharide treated female mice, whereas in males, no increased BBB permeability for selenite was observed under such conditions (Minami et al., 2002). The sex-specific phenotype in

Se metabolism is rather well-described – the reader is referred to the review by Seale et al. (2018b). Additionally, Se treatment (100 nmol/L) was shown to inhibit glucose-induced expression of adhesion molecules in the human umbilical vein endothelial cells (Zheng et al., 2008); however, the research in brain endothelium is still required to evaluate the role of Se in cell adhesion in BBB and BCB. Oztas et al. (2007) reported that sodium selenite (4 ppm in rat dams drinking water) and vitamin E supplementation had a beneficial effect on BBB integrity in the rat pups. However, the studies on the effect of dietary Se and/or selenoprotein expression on, e.g., tight junction protein expression or systemic *in vivo* research on Se/selenoprotein in BBB and BCB permeability are currently absent, to the best of the author's knowledge.

CONCLUSION AND PERSPECTIVES

The understanding of brain Se transport has considerably improved, first of all, over the past two decades. The primary SELENOP/LRP8-dependent mechanism of Se entering the brain is rather well described. However, even in this respect, there are still some gaps remaining. For instance, ApoE, sharing the brain uptake receptor with SELENOP, appears to be important for the regulation of tight junction integrity at the BBB (Blanchette and Daneman, 2015). This is tempting to speculate on possible interplay, but such speculations require a further warrant, especially *in vivo* research.

Another currently understudied and important aspect of Se interplay with BBB and BCB may be accomplished through immune and inflammatory pathways. Individual selenoproteins are known to be involved in regulating inflammation and immunity. Se deficiency negatively impacts immune cells during activation, differentiation, and proliferation through redox signaling, oxidative burst, calcium flux, and the subsequent effector functions of immune cells (Huang et al., 2012; Avery and Hoffmann, 2018; Toledo et al., 2020). For instance, the potential inhibition of the nuclear factor kappa-B (NFκB) signaling pathway by Se and selenocompounds is often considered (Santamaría et al., 2005; Vunta et al., 2007; Duntas, 2009; Gholami et al., 2015). Importantly, Dreher et al. (1997) demonstrated *in vitro* that human SELENOP gene's promoter was cytokine responsive. Consequently, inflammatory processes may affect SELENOP production by the liver thus influencing brain Se uptake.

Se metabolism is known to be affected by sex (Schomburg, 2016; Seale et al., 2018b). On the other hand, common neurological diseases, e.g., Alzheimer's disease have pronounced both marked sex-dependency (Ferretti et al., 2018) and involvement in the BBB decline (Zenaro et al., 2017). Additionally, studies in mice have shown higher liver and kidney expression of *Selenop* in females than that in males (Schomburg et al., 2007), so SELENOP/LRP8-brain Se uptake pathway may also exert sex-dependence. Thus, another aspect for future research of Se at the brain barriers may be related to improving our understanding on sex-related differences, including the effects of dietary Se, selenoproteins, and selenometabolites on permeability and function of BBB, BCB as well as gut-brain axis and brain immunity. The same corresponds to the effect of age and aging on the brain barrier functions and the related effects of Se and selenoproteins (Huang et al., 2012).

Many studies indicate the critical importance of the exact chemical speciation of essential trace elements (Templeton et al., 2000) in determining their biological activity (Michalke et al., 2018). For instance, in *post mortem* studies in the human brain from Alzheimer's disease patients, it was demonstrated that the Se distribution pattern in the brain is seriously distorted (Bellinger et al., 2008; Rueli et al., 2015). SELENOP was shown to be co-localized with Alzheimer's disease brain tissue lesions – Aβ plaques and neurofibrillary tangles (Bellinger et al., 2008). Moreover, in a further study of the same group, the increased release of SELENOP from the *choroid plexus* to the CSF in Alzheimer's disease patients was reported (Rueli et al., 2015). Speciation studies in CSF demonstrated that exposure of the brain tissue to hexavalent Se may be involved in Alzheimer's (Vinceti et al., 2017a, 2019) or amyotrophic lateral sclerosis pathology (Vinceti et al., 2013b; Mandrioli et al., 2017; Violi et al., 2020). Unfortunately, the exact molecular pathways of Se in the neurodegenerative processes, including the transport through the neural barrier endothelia have only been studied scarcely. The diverse biological activities of Se urgently require systematic studies concerning its behavior at the BBB and BCB and its role in maintaining barrier function and integrity. The investigations on *in vitro* BBB or BCB models, analogous to those published by Bornhorst et al. (2012) and Müller et al. (2018) for other elements could help to further clarify barrier processes regarding Se and seleno-species. Mapping of brain-barrier regions with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) technology for Se in combination with microscopy and histology is encouraged to support this interesting research field. Recent technological advances in analytical science are now enabling the study of Se transport, its spatial and chemical distribution at an unprecedented level of detail. Finally, the question of Se efflux from the brain is not properly addressed. It should be noted that uptake of Se into the brain compartment without a balanced Se-discharge mechanism could finally lead to local, brain-compartment related Se-overexposure. In contrast to this necessary balance, there is a considerable misbalance between the studies concerning Se entering the brain or particular brain cells and these on Se leaving the CNS. Along with thorough literature research on studies about Se-efflux from brain compartment no references were found. Although similar mechanisms may be involved in Se discharge from the brain, i.e., involving SELENOP and minor low molecular weight Se-metabolites, there are no relevant studies supporting this notion, to the best of the authors' knowledge. That misbalance should be addressed in future research.

AUTHOR CONTRIBUTIONS

NS and BM conceived the agenda for the review. NS contributed to all sections. ED largely contributed to section "Blood–Brain Barrier, Blood–Cerebrospinal Fluid Barrier, and

Neurovascular Unit" and prepared all the figures. BB mainly contributed to sections "Introduction," "Blood–Brain Barrier, Blood–Cerebrospinal Fluid Barrier, and Neurovascular Unit," and "Selenium Transport to The Brain – Selenoprotein P and Low Molecular Weight Selenium-Species." BM mainly contributed to sections "Introduction," "Selenium Transport to The Brain – Selenoprotein P and Low Molecular Weight Selenium-Species," and "Conclusion and Perspectives." All authors contributed to the editing and discussion and agreed to submit the manuscript in its current state.

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The Neurobiology of Selenium: Looking Back and to the Future

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Eighteen years ago, unexpected epileptic seizures in *Selenop*-knockout mice pointed to a potentially novel, possibly underestimated, and previously difficult to study role of selenium (Se) in the mammalian brain. This mouse model was the key to open the field of molecular mechanisms, i.e., to delineate the roles of selenium and individual selenoproteins in the brain, and answer specific questions like: how does Se enter the brain; which processes and which cell types are dependent on selenoproteins; and, what are the individual roles of selenoproteins in the brain? Many of these questions have been answered and much progress is being made to fill remaining gaps. Mouse and human genetics have together boosted the field tremendously, in addition to traditional biochemistry and cell biology. As always, new questions have become apparent or more pressing with solving older questions. We will briefly summarize what we know about selenoproteins in the human brain, glance over to the mouse as a useful model, and then discuss new questions and directions the field might take in the next 18 years.

Keywords: genetics, neurodegeneration, GPX4, ferroptosis, epilepsy

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A BRIEF HISTORY OF THE FIELD

When selenoprotein P (*Selenop*)-knockout mice were made independently in two laboratories, it was not expected that their most dramatic phenotype was to reveal the essential requirement of selenium (Se) in the brain (Hill et al., 2003; Schomburg et al., 2003). However, the model was tricky: the neurological phenotype depended exquisitely on the level of dietary Se supply and, more precisely, on the timing of Se deficiency during the ontogeny of the animal. The neurological phenotype varied between none at all, epileptic seizures, movement phenotype with ataxia and/or dystonia, overt neurodegeneration with premature death, or death before weaning (Hill et al., 2004; Schweizer et al., 2004b, 2005; Valentine et al., 2005; Schweizer, 2016). Since Se levels in commercial diets may not always precisely reflect the printed specifications in every lot of mouse chow, and because the *Selenop*-knockout mice reflected so sensitively the dietary Se supply, working with the model was difficult, to say the least. At the time, antibodies against selenoproteins were not widely available, and enzymatic assays for glutathione peroxidase (GPX) and thioredoxin reductase (TXNRD), Se measurements, and metabolic labeling with ^{75}Se were the methods of choice. At least, these methods clearly demonstrated, that inactivation of *Selenop*, a gene mostly expressed in liver and secreted into the plasma, reduced Se levels and selenoenzyme activities in the brain to a degree impossible to achieve with dietary Se restriction alone (Hill et al., 2003; Schomburg et al., 2003). New selenoproteins were still being discovered, until a landmark genomic study fixed the number

at the final 25 in humans and 24 in mice (Kryukov et al., 2003). In an early review (Schweizer et al., 2004c), the key questions about the neurobiology of Se were: Which selenoproteins are expressed in the brain, in which regions and in which cell types? What are their functions? How does Se enter the brain and is it distributed in a hierarchical manner? Is there a causal connection to neurological disorders?

To cut the answers short: most selenoproteins are expressed in the brain, mainly in neurons, in all major brain regions (Zhang et al., 2008). Gene targeting in mice for all or single selenoproteins has revealed that GABAergic interneurons are particularly vulnerable, but also basal ganglia, cerebellum, cortex, and brain stem (Valentine et al., 2005; Wirth et al., 2010, 2014; Pitts et al., 2012; Seeher et al., 2014). At first it remained an open question whether myelination defects were caused by neuronal or oligodendroglial selenoprotein dysfunction (Valentine et al., 2008). Several selenoproteins are essential for the brain development and function, in particular glutathione peroxidase 4 (GPX4), but also thioredoxin reductase 1 (TXNRD1), and SELENOT (Castex et al., 2015). Currently the function of GPX4 as a major regulator of ferroptosis in development and disease is receiving a great deal of attention (Stockwell et al., 2017; Friedmann Angeli et al., 2019). It will be seen whether GPX4 is an indispensable protein for neurons in its own right or whether there is a causal connection to neurodegeneration in neurological disorders. What is now clear, is that Se enters the brain either as non-physiological selenite salt or, more physiologically, in the form of SELENOP, loaded in the liver with several atoms of Se (Schweizer et al., 2005; Renko et al., 2008; Hill et al., 2012), and taken up at the blood-brain-barrier (BBB) and by individual cells via endocytosis using receptors of the LRP family (Burk and Hill, 2015). The most important receptor is APOER2 (Olson et al., 2007), but also LRP2/MEGALIN (Olson et al., 2008; Chiu-Ugalde et al., 2010) and, most likely, LRP1 contribute to SELENOP internalization. Among these receptors, APOER2 clearly is the most important, providing preferential Se supply at the BBB and in the testis. MEGALIN appears more involved in Se supply through the blood-cerebrospinal fluid barrier and in the kidney (Burk and Hill, 2015). *SELENOP* is also expressed in the brain and may contribute to local Se storage and recycling (Scharpf et al., 2007; Renko et al., 2008). SELENOP and its receptors contribute to a "hierarchy" of selenoprotein expression in organs in the sense that some organs, like the brain, can be preferentially supplied with Se at the expense of others, like the liver. There is, however, a second "hierarchy" of selenoprotein expression that depends on the relative sensitivity of individual selenoproteins to Se availability. Thus, GPX1 is more sensitive to cellular Se levels than, e.g., GPX4. The reasons behind this observation are multifarious and beyond the scope of this review. Both hierarchies may work hand-in-hand as *GPX1* mRNA is highly expressed in liver and contains a highly efficient SECIS element. Depending on dietary Se supply, the amount of GPX1 protein covers orders of magnitude, and likely provides a safe Se storage device, until the Se is needed for distribution via SELENOP to preferred target tissues. The amount of Se in a tissue does not necessarily inform about its physiological importance, e.g., brain Se levels are much lower than liver Se levels, yet selenoproteins are essential in brain, but not liver (Schweizer et al., 2005; Wirth et al., 2010).

GENETIC DEFICIENCY OF SINGLE SELENOPROTEINS

At that time, only one human disorder was genetically linked to deficiency of a selenoprotein-encoding gene, now known as selenoprotein N (SELENON)-related myopathies (Moghadaszadeh et al., 2001; Castets et al., 2012), but the situation was soon to change with exome sequencing entering clinical practice. SELENON is an ER-resident membrane protein of unknown function. Mutations in *SELENON* that disrupt the gene or prevent selenocysteine (Sec) insertion into the protein lead to myopathy (Villar-Quiles et al., 2020). Mouse and zebrafish models of SELENON-deficiency reflect aspects of the muscular phenotype, in particular the preferential affection of axial muscles (Rederstorff et al., 2011). This example suggests that the mouse may represent an acceptable model for humans regarding selenoprotein deficiency. It should be noted that dietary deficiency for Se in livestock was recognized early as a cause for white muscle disease (Muth et al., 1958). So far there is no evidence to link *SELENON* mutations to impaired neuronal or neurological function.

The syndromes associated with selenoprotein deficiency can be broadly divided into two categories: the first category represents mutations in single genes encoding selenoproteins. The second category is represented by mutations in genes involved in selenoprotein biosynthesis. A comprehensive compilation of individual mutations and patient phenotypes can be found in recent summaries of the state of the field (Schweizer and Fradejas-Villar, 2016; Fradejas-Villar, 2018; Schoenmakers and Chatterjee, 2020). Here, we will only briefly summarize these results and rather focus on new developments since then.

Conditional gene inactivation of *Gpx4* in mice demonstrated that GPX4 is an essential selenoprotein for several types of neurons as discussed above (Seiler et al., 2008). Conditional inactivation of *Gpx4* in forebrain neurons after development lead to cognitive decline and hippocampal neurodegeneration (Hambright et al., 2017). Furthermore, constitutive gene inactivation of *Gpx4* lead to embryonic lethality around embryonic day 7 (Yant et al., 2003; Seiler et al., 2008). Thus, it came as a complete surprise to find newborn children affected with Sedaghatian-type spondylometaphyseal dysplasia with inactivating non-sense mutations in the *GPX4* gene (Aygun et al., 2012; Smith et al., 2014). These patients show massive brain atrophy and usually die shortly after birth. In stark contrast to mice, where *Gpx4*-deficiency leads to early embryonic lethality, human fetuses obviously progress much further in their development.

A lesser sensitivity of humans compared to mice regarding the lack of selenoproteins was also observed with respect to TXNRD2. *Txnrd2*$^{-/-}$ mice died around embryonic day 13 with thinned ventricular walls in the heart and impaired hematopoiesis (Conrad et al., 2004). Conditional ablation of

Txnrd2 in the heart lead to a fatal cardiomyopathy (Conrad et al., 2004). Similarly, heterozygous missense mutations were associated with dilated cardiomyopathy in humans (Sibbing et al., 2011) and were reminiscent of Keshan disease, a fatal cardiomyopathy observed in Se-deficient regions in China (Ge et al., 1983; Loscalzo, 2014). It thus came as a complete surprise that a homozygous truncating mutation in human *TXNRD2* merely resulted in familial glucocorticoid deficiency without a cardiac phenotype (Prasad et al., 2014).

Similar to inactivation of *Txnrd2*, constitutive inactivation of *Txnrd1* is embryonic lethal in mice (Jakupoglu et al., 2005). Conditional ablation of *Txnrd1* in neuronal precursors shows only a mild cerebellar defect (Soerensen et al., 2008), while neuron-specific *Txnrd1* ablation leads to neurodegeneration with aging (Schweizer and Schomburg, 2006). Since our last review of this subject, we have found that homozygous mutations in *TXNRD1,* which reduce enzymatic activity, are associated with genetic generalized epilepsy in human (Kudin et al., 2017). In summary, upon *Txnrd* mutation the mouse phenotypes appear throughout more severe than the corresponding human phenotypes. This raises the question whether variants of TXNRDs may be able to compensate for the loss of the other TXNRD in humans, but not in mice, or whether, e.g., the glutaredoxin system may be able to partially compensate in some human cell types.

Ethanolamine-phosphotransferase 1 (EPT1/SELENOI) is one of two enzymes catalyzing the same step in phospholipid biosynthesis (Gladyshev et al., 2016). This enzyme is obviously important for myelin biosynthesis in the human brain (Ahmed et al., 2017; Horibata et al., 2018). Inactivation of the gene in mice is embryonic lethal (Avery et al., 2020). Se deficiency of the brains of *Selenop*-deficient mice impaired the myelin sheath in the brain stem, at least under conditions of low Se diet (Valentine et al., 2005). Thus, it is possible that the myelination defect is a primary phenotype of myelin formation in oligodendrocytes and not a result of retrograde signaling from selenoprotein-deficient neurons, as we initially suspected.

The only other selenoprotein that has been shown to play an essential role in the brain is SELENOT (Castex et al., 2015; Boukhzar et al., 2016). How this relates to its role in protein glycosylation is not clear, yet (Hamieh et al., 2017). It is intriguing, that other selenoproteins have also been implicated in protein glycosylation or protein folding, e.g., SELENOF and SELENOM. The respective knockout mouse models, however, did not show any apparent neurological defects (Kasaikina et al., 2011). SELENOS, another selenoprotein implicated in endoplasmic reticulum associated degradation of proteins (ERAD) (Curran et al., 2005), has not been studied by gene targeting in mice. While selenoproteins have often simply been classified as "anti-oxidant," it is remarkable that inactivation of a selenoenzyme with a defined reductase activity, methionine-R-sulfoxide-reductase B1 (MSRB1), has not produced a neurological phenotype (Fomenko et al., 2008; Lee et al., 2013).

There is a rich literature on mouse models of neurodegenerative diseases whose phenotypes can be exacerbated by additional deficiency of "antioxidant" selenoproteins (Schweizer et al., 2004a; Zhang et al., 2020). In such models there is always the conceptual question whether the mutation in the selenoprotein specifically abrogates (and thus reveals) a specific protective mechanism or whether the selenoprotein mutation simply tips over a dysbalanced system that is already vulnerable to any other possible stressor. Given the availability of many powerful genome-wide association studies on important neurodegenerative disorders, and their failure to identify mutations in selenoprotein genes, it seems unlikely for us that mutations in selenoproteins are important causes or modifiers of common neurological disorders. Yet, mutations in selenoproteins or their biosynthesis pathways may reveal specific cell biological or developmental functions of selenoproteins.

SELENOPROTEIN DEFICIENCY RESULTING FROM MUTATIONS IMPAIRING SELENOPROTEIN BIOSYNTHESIS

A landmark paper on the identification of mutations in the selenoprotein biosynthesis factor SECISBP2 in humans called into question the possibly simple-minded concept of selenoproteins as "anti-oxidants." The key phenotype that brought the patients to medical attention, was a growth retardation in puberty (Dumitrescu et al., 2005). Abnormal thyroid function tests (TFT), i.e., the constellation of thyroid-stimulating hormone (TSH) and thyroid hormone levels, guided the discovery of a congenital deficiency of selenoprotein biosynthesis. The pubertal growth spurt depends not only on growth hormone, but requires permissive action of thyroid hormone. The TFT suggested deficiency of deiodinase 2 (DIO2) activity in these patients which was confirmed in patient fibroblasts. Deiodinases are selenoenzymes capable of removing iodide from iodothyronines (Köhrle et al., 2005; Mondal et al., 2016). The prohormone thyroxine (T4) requires 5′-deiodination to yield triiodothyronine (T3), which binds the nuclear T3-receptors (**Figure 1**). 5-deiodination of T4 and T3 yields reverse T3 (rT3) and 3,3′-T2, respectively. Moreover, the two plasma selenoproteins SELENOP and GPX3 were reduced in these patients (Dumitrescu et al., 2005). Thus, the congenital deficiency of selenoprotein biosynthesis revealed itself not in neurodegeneration, epilepsy, heart disease or a muscular disorder, but in altered thyroid hormone levels in the sense of a blunted response to T4! Later, more patients with apparently stronger mutations in SECISBP2 were identified (Di Cosmo et al., 2009; Azevedo et al., 2010; Schoenmakers et al., 2010). Some of these patients exhibited a SELENON-related myopathy, infertility, and an immune phenotype (Schoenmakers and Chatterjee, 2018). The importance of local conversion of T3 is illustrated by the Thr92Ala polymorphism in DIO2. People with the homozygous Ala92 version of this polymorphism have a reduced ability to convert T4 to T3 (McAninch et al., 2015), hence when being treated for hypothyroidism, have improved psychological well-being on combination T4/T3 therapy than on T4 treatment alone (Bianco and Kim, 2018). Remarkably, the first patient with a mutation in the $tRNA^{Sec}$ gene (*TCA-TRU* in human, *Trsp* in mouse) showed the same phenotype of a blunted response to T4 (Schoenmakers et al.,

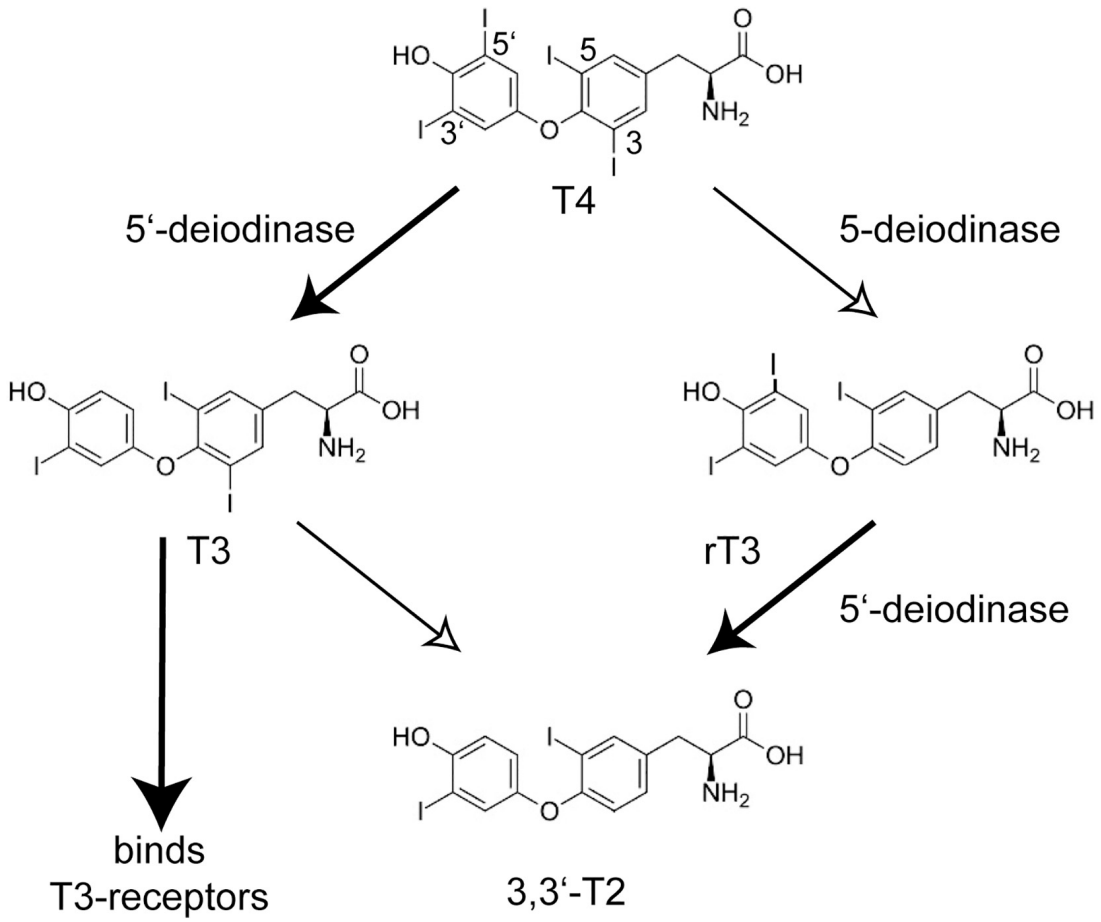

FIGURE 1 | Conversion of iodothyronines by deiodinases and activation of the nuclear T3-receptors. The main product of the thyroid gland is thyroxine (T4, 3,3′,5,5′-tetraiodothyronine). The actions of 5′-deiodinases (DIO1 and DIO2) and 5-deiodinases (DIO1 and DIO3) lead to T3 (3,3′,5-triiodothyronine) and rT3 (3,3′,5′-triiodothyronine), respectively. Only T3 activates the nuclear T3-receptors TRα and TRβ. T2 and rT3 are thus inactive metabolites and a cell can shape its local T3 level through DIO expression. Inactivation of *Dio1* as well as *SECISBP2*-deficiency lead to increased plasma rT3.

2016). Findings from *Dio1*- and *Dio2*-knock-out mouse models are entirely compatible with the above conclusions drawn from *SECISBP2*-deficiency (Schneider et al., 2001, 2006). Selenoprotein deficiency does not fundamentally impair thyroid gland function (Chiu-Ugalde et al., 2012).

This leads us to the obvious question whether other factors involved in selenoprotein biosynthesis have been found mutated in humans. And what kind of phenotypes are presented by affected individuals? The first patients with mutations in the selenocysteine synthase gene (*SEPSECS*) have been identified in Agamy et al. (2010). The patients presented with "progressive cerebello cerebral atrophy," now systematically designated pontocerebellar hypoplasia 2D (PCH2D). The names of the syndromes capture quite well the observed phenotypes (Schoenmakers and Chatterjee, 2020). The predominantly neurological condition with neurodegeneration and epilepsy is likely based on dysfunction of GPX4 and other essential selenoproteins, possibly TXNRD1 or 2 (Anttonen et al., 2015). Some patients display milder phenotypes and may grow into adulthood with intellectual disability, but no overt neurodegeneration (Iwama et al., 2016). Interestingly, we are not aware of reports of abnormal TFT in these patients. Likewise, we do not know of a *SELENON*-related myopathy in one of these patients.

Individuals with mutations in *EEFSEC*, *SEPHS2*, and *PSTK* have not yet been found (**Figure 2**). Selenoprotein expression in knockout mouse models for these genes have not been described. Conditional inactivation of the suggested biosynthesis factor SECP43, encoded by the *Trnau1ap* gene, in liver and in neurons did not support a role for this gene in selenoprotein expression (Mahdi et al., 2015). Mouse models for hypomorphic mutations in the $tRNA^{Sec}$ have been generated. One model has a mutation in the promoter and, as a simple transgene, is inserted somewhere in the genome (Carlson et al., 2009). This mouse displays a neurological phenotype that resembles in several aspects of *Selenop*- and neuron-specific *Trsp*-knockout mutants (Schomburg et al., 2003; Wirth et al., 2010, 2014). Point mutations have been made in *Trsp* affecting the anticodon loop of $tRNA^{Sec}$. Overexpression of the A37G-*Trsp* mutant (made as a simple transgene) resulted in neurological defects, in particular

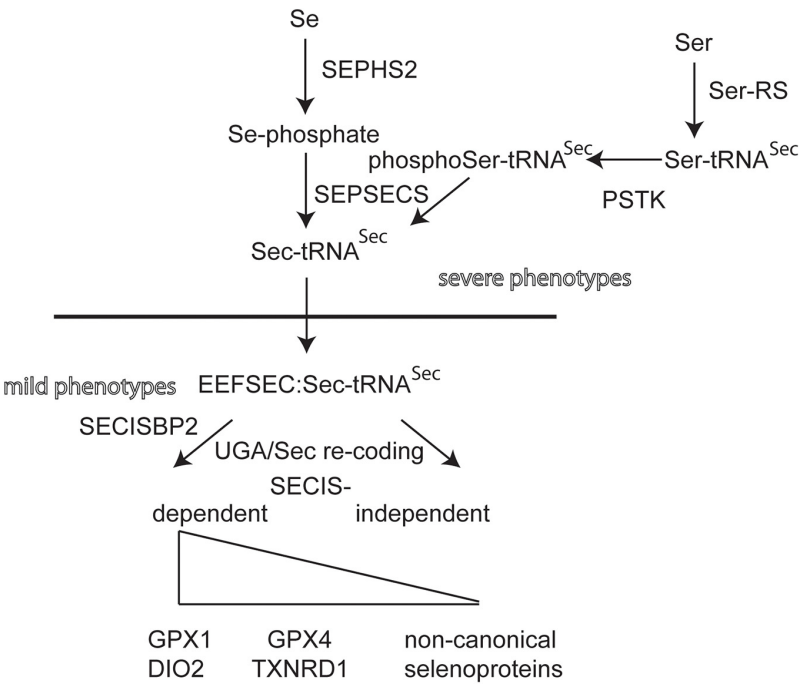

FIGURE 2 | Biosynthetic pathway of selenoprotein translation. Transfer-RNA^{Sec} is charged with Ser by Seryl-tRNA synthase (Ser-RS), hence the more accurate designation tRNA$^{[Ser]Sec}$. The kinase PSTK phosphorylates Ser-tRNA^{Sec}. Selenophosphate synthase (SEPHS2) provides selenophosphate which is used by selenocysteine synthase (SEPSECS) to convert phosphoSer-tRNA^{Sec} into Sec-tRNA^{Sec}. EEFSEC is a translation elongation factor specific for Sec-tRNA^{Sec}. Canonical selenoproteins carry a selenocysteine insertion sequence (SECIS) in their mRNA in order to re-code the UGA codon as Sec codon. The dependence of UGA/Sec re-coding varies among canonical selenoproteins. Several non-canonical selenoprotein genes have been described that do not contain a SECIS element (Guo et al., 2018).

when fed a high Se diet (Kasaikina et al., 2013). TFTs have not been determined for the *Trsp* mutant mouse models.

Looking at these results, there seems to exist a strange dichotomy of phenotypes being related either to neurobiology or endocrinology, when selenoprotein biosynthesis is impaired. This observation holds for both mice and humans. A naïve epistatic model of selenoprotein biosynthesis should predict more or less the same phenotypes, if selenoprotein translation is globally impaired (**Figure 2**). Clearly, we have not yet analyzed all possible mutants and not all available mutants have been systematically analyzed side by side. Yet, in humans, mutations most often come as missense, splicing or other mutations that may not completely abrogate 100% of gene/protein expression/activity. We have recently shown that the effect of a missense mutation *in vitro* and *in vivo* may differ, in particular stability of a mutated protein may depend on the cell type (Zhao et al., 2019). For the SECISBP2^{R543Q} mutant, we demonstrated that the protein is a complete *NULL* in mouse liver, but partially functioning and supporting selenoprotein expression in neurons (Zhao et al., 2019).

FUTURE DIRECTIONS

In order to understand the neurobiology of Se, we need both, the precise biochemical or cell biological function of each selenoprotein and the full understanding of the phenotypes under conditions of its absence in an entire mammalian organism. This goal has only been achieved for a small subset of selenoproteins. For some of the others, we may have a biochemical reaction and a phenotype of cells grown in a dish, but we are convinced that nobody would have been able to predict the complex phenotype of patients with mutations in *SECISBP2* based on the finding of reduced selenoprotein expression in *SECISBP2*-deficient cells in culture. If we just focus on the brain with its many neuronal and glial cell types, we are confronted with perplexing complexity (Zhang et al., 2008). All of these cell types are involved in mechanisms of development, exert a function in the mature organism, and may play are role in neurodegeneration. Thus, it is obvious how wide this field still is and how much expertise is required to address this question.

Another question related to the discussion above, is to what extent mice are valid models for humans with regard to understanding the functions of selenoproteins. The answer will again rely on the comparison of genetic models in mice and patients with congenital defects in selenoprotein genes or genes encoding selenoprotein biosynthesis factors. We can expect that exome-sequencing approaches that are now broadly available will help us identify patients with such mutations. A recent thought-provoking paper has looked at the same question from just the opposite perspective: Instead of searching for mutations in the genes of patients with clinical phenotypes, Santesmasses et al. (2020) searched human genome data for inactivating

mutations in selenoprotein genes. They found that humans can carry homozygous inactivating mutations in *SELENOO* without apparently presenting with a phenotype. SELENOO is a novel mitochondrial protein Ser/Thr-AMP transferase that has not yet been inactivated in mice (Sreelatha et al., 2018).

The question whether the selenoproteome is completely known seemed to have been solved through the landmark paper by Kryukov et al. (2003) who identified genes encoding selenoproteins based in part on the presence of the SECIS element. A recent proteome paper now suggested there are additional Sec-containing proteins with UGA/Sec codons, but lacking recognizable SECIS elements (Guo et al., 2018). This provocative finding is, interestingly, in line with the demonstration of selenoprotein translation in the absence of functional SECISBP2 (Fradejas-Villar et al., 2017; Zhao et al., 2019). If mutations in *SEPSECS*, unlike mutations in *SECISBP2*, would also affect selenoproteins that do not depend on a SECIS for biosynthesis, the dichotomy of phenotypes could be explained and some of the non-canonical selenoproteins would likely be important for the brain.

The arguably most dynamic field of selenoprotein research, again related to the neurobiology of Se, is the wider context of the function of GPX4. The whole field of ferroptosis is blossoming. This type of cell death emerges as an important cell biological process on which much hope is placed in the context of cancer treatment and prevention of neurodegenerative disease. Can ferroptosis be modulated pharmacologically to the benefit of patients? Do other pathways related to selenoproteins play a role in these processes? What is the role of lipid peroxidation in this context? In mitochondria? This reminds one of us (US) of a lab rotation in organic chemistry long ago: during his undergraduate study he separated lipid-hydroperoxides and their respective alcohols on a chiral gas-chromatographic column and observed that the peroxides and alcohols were chiral. An enzymatic process seemed the most likely explanation, while biologists argued that spontaneous lipid peroxidation was most likely overinterpreted. . . Keeping this in mind, who knows what exciting findings there are just around the corner revealing a part of themselves seemingly as oddities or artifacts?

AUTHOR CONTRIBUTIONS

US wrote the initial draft of the manuscript. All authors refined and rewrote parts of the manuscript and contributed to research associated with the thoughts presented here.

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REVIEW
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Roles of Selenoproteins in Brain Function and the Potential Mechanism of Selenium in Alzheimer's Disease

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Selenium (Se) and its compounds have been reported to have great potential in the prevention and treatment of Alzheimer's disease (AD). However, little is known about the functional mechanism of Se in these processes, limiting its further clinical application. Se exerts its biological functions mainly through selenoproteins, which play vital roles in maintaining optimal brain function. Therefore, selenoproteins, especially brain function-associated selenoproteins, may be involved in the pathogenesis of AD. Here, we analyze the expression and distribution of 25 selenoproteins in the brain and summarize the relationships between selenoproteins and brain function by reviewing recent literature and information contained in relevant databases to identify selenoproteins (GPX4, SELENOP, SELENOK, SELENOT, GPX1, SELENOM, SELENOS, and SELENOW) that are highly expressed specifically in AD-related brain regions and closely associated with brain function. Finally, the potential functions of these selenoproteins in AD are discussed, for example, the function of GPX4 in ferroptosis and the effects of the endoplasmic reticulum (ER)-resident protein SELENOK on Ca^{2+} homeostasis and receptor-mediated synaptic functions. This review discusses selenoproteins that are closely associated with brain function and the relevant pathways of their involvement in AD pathology to provide new directions for research on the mechanism of Se in AD.

Keywords: selenoprotein, neurotransmission, brain function, Alzheimer's disease, Ca^{2+} homeostasis

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INTRODUCTION

Over the past decades, selenium (Se) and its compounds have mainly been the focus of research on regulation of development and the immune system and on antitumor properties due to their strong antioxidant activities (Rayman, 2000; Schomburg, 2016). Nutritional data show that under normal diet conditions, the Se level is highest in the kidney, followed by the liver, spleen, pancreas, heart, and brain (Chen and Berry, 2003). However, when Se uptake is insufficient, this ranking changes according to the priority order of different organs for Se. Among organs, the brain retains Se the longest (Burk et al., 1972; Brown and Burk, 1973), indicating the importance of Se in the maintenance of physiological function in the central nervous system (CNS). Epidemiological surveys show a significant positive correlation between the Se level and cognitive ability—i.e., a

dose-response effect (Gao et al., 2007)—and that the blood Se level gradually decreases with age (Berr et al., 1993). Furthermore, Se levels change significantly in the brain and blood of patients with various neurodegenerative diseases [such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, and Batten's disease] (Chen and Berry, 2003). Therefore, the role of Se in the brain and in neurodegenerative diseases has gradually become a research hotspot.

AD is an age-related neurodegenerative disease with a very high prevalence among elderly people. The Se level in AD patients and carriers of the apolipoprotein E (ApoE4) allele (a high risk factor for sporadic AD) is significantly decreased (Cardoso et al., 2010), suggesting that Se deficiency is associated with AD. Senile plaques generated by β-amyloid (Aβ) deposition and neurofibrillary tangles (NFTs) formed by tau hyperphosphorylation in the brain are two major pathological features of AD. Early studies showed that Se supplementation in SH-SY5Y cells expressing a mutant amyloid precursor protein (APP) gene reduced lipid peroxidation product levels, inhibited β-secretase 1 (BACE1), and γ-secretase activity, and reduced Aβ aggregation (Gwon et al., 2010). Sodium selenate, an inorganic Se compound, was once considered a useless Se compound because of its low bioavailability (Daniels, 1996). Interestingly, van Eersel et al. (2010) and Jin et al. (2017) used transgenic animal models of AD to show that sodium selenate reduced tau protein phosphorylation and ameliorated cognitive impairment in AD mice by regulating the activity of protein phosphatase 2A (PP2A) (Ishrat et al., 2009; Lovell et al., 2009; Corcoran et al., 2010; van Eersel et al., 2010; Jin et al., 2017), which undoubtedly promoted research regarding Se in AD prevention and treatment. Organic Se has attracted attention due to its advantages, including its enhanced biological activity, decreased toxicity, and diminished environmental pollution concern compared to inorganic Se. Many organic Se compounds have been shown to have therapeutic effects in AD mice models (Xie et al., 2017, 2018; Zhang et al., 2017a, 2018). Through a series of studies, Zhang et al. showed that selenomethionine enhanced the antioxidant capacity, mitigated Aβ and tau pathology, reversed synaptic deficits, and ameliorated cognitive decline in AD mice (Song et al., 2014; Zhang et al., 2016, 2017a, 2018), demonstrating the multitarget effect of Se in AD treatment. Furthermore, impaired autophagy (Zhang et al., 2017b,c; Song et al., 2018) and mitochondrial dysfunction (Balaban et al., 2017) have been shown to be the targets of Se compounds in AD. However, the in-depth mechanisms of Se in AD prevention and treatment remain unclear, and this lack of knowledge is a major factor limiting further clinical application of Se in AD and a reasonable explanation for the unsuccessful randomized clinical trial of Se in the AD study of Kryscio et al. (2017), which was based on the Se and vitamin E Cancer Prevention Trial.

Though there are several Se utilization pathways in the body, selenoprotein synthesis is the main means by which Se exerts numerous biological functions. Currently, genes encoding 25 selenoproteins have been identified in human genomic sequences (Kryukov et al., 2003). Glutathione peroxidase (GPX) was the first identified selenoprotein and participates in redox reactions. Se-containing GPX isozymes (GPX1, GPX2, GPX3, GPX4, and GPX6) exhibit different tissue-specific expression patterns and subcellular localization (Brigelius-Flohe, 1999). Thioredoxin reductases (TrxRs) (TXNRD1, TXNRD2, and TXNRD3) are other antioxidases that contribute not only to the antioxidant system but also to cell proliferation and apoptosis (Mustacich and Powis, 2000). Thyroid hormone deiodinases (DIOs) (DIO1, DIO2, and DIO3) participate in T3 and T4 production and regulate thyroid hormone activities (Kohrle, 2000). Methionine-sulfoxide-reductase 1 (MSRB1), also called SELENOR, is responsible mainly for repairing methionine-oxidized proteins. Interestingly, selenophosphate synthetase 2 (SEPHS2) (an enzyme involved in selenocysteine (Sec) biosynthesis) is also a selenoprotein. As a plasma Se transport protein, SELENOP also exhibits lipid hydroperoxide reductase activity (Saito, 2020). SELENOK, SELENOM, SELENOF, SELENOS, SELENOT, DIO2, and SELENON are endoplasmic reticulum (ER)-resident selenoproteins and participate mainly in the regulation of physiological processes, including Ca^{2+} flux, protein folding, and ER stress (Pitts and Hoffmann, 2018). SELENOO, the largest mammalian selenoprotein, possesses a protein kinase-like domain and may have a function in oxidative stress response (Lenart and Pawlowski, 2013). Sporadic reports have addressed other selenoproteins (SELENOW, SELENOH, SELENOI, SELENOU, and SELENOV), which still lack clear recognition except for their antioxidant function.

Biochemical and bioinformatic analyses have shown that most selenoproteins are expressed in the brain (Zhang et al., 2008) and that some selenoproteins are closely associated with brain function. The tRNA[Ser]Sec (*Trsp*) gene is required for the expression of all functional selenoproteins. Neuron-specific *Trsp*-knockout mice had significantly decreased expression levels of selenoproteins in the brain and showed delayed growth, loss of balance, and extensive neuronal degeneration (Wirth et al., 2010). Additionally, Selenop$^{-/-}$ mice exhibited many features of neurological dysfunction (Hill et al., 2004; Schweizer et al., 2005). GPX4-regulated ferroptosis can induce progressive cognitive impairment and hippocampal neurodegeneration in mice (Hambright et al., 2017). Thus, these brain function-related selenoproteins may be involved in the occurrence and development of AD. However, the selenoproteins involved and their roles in these processes remain unclear. This review analyzes the expression and distribution of selenoproteins in the brain, assesses the associations between various selenoproteins and brain function and the potential of these selenoproteins in AD research, and discusses the roles of these selenoproteins in AD pathology.

Expression and Distribution of Selenoproteins in the Brain

Insertion of UGA-encoded Sec into a translating selenoprotein polypeptide is a complex and sophisticated protein translation process controlled synergistically by multiple regulatory factors (Bulteau and Chavatte, 2015). Differential expression levels of selenoproteins in tissues and organs are directly associated with the biological functions of selenoproteins. The current data regarding the differential expression levels of various

selenoproteins in the brain originated from the bioinformatic studies conducted by Zhang et al. (2008). The transcripts of 24 selenoproteins are expressed in the mouse brain. The expression levels of GPX4, SELENOK, SELENOM, SELENOW, and SELENOF in the brain are higher than those of other selenoproteins, and GPX4, SELENOP, and SELENOW are expressed at high levels in more than 90% of brain regions. A recent gene transcriptomic analysis (Fagerberg et al., 2014) ranked the expression levels of 25 selenoproteins in the human brain (**Figure 1**). Among all selenoproteins, 6 (GPX3, DIO3, GPX2, DIO1, SELENOV, and GPX6) were confirmed to have very low or no expression in the human brain. SELENOW, GPX4, SELENOP, SELENOF, and SELENOK have the five highest expression levels, and this pattern is basically consistent with that observed in the mouse brain (Zhang et al., 2008). The next most highly expressed selenoproteins, in decreasing order, are SELENOT, SELENOH, GPX1, TXNRD1, SELENON, SELENOS, SEPHS2, SELENOI, and SELENOM. Six ER-resident selenoproteins (except for DIO2), especially SELENOF, SELENOK, and SELENOT, are expressed at relatively high levels in the brain. In addition, the selenoenzymes GPX4, GPX1, TXNRD1, and SEPHS2 are expressed at high levels in the brain. SELENOP is highly expressed in the brain due to its Se transport function. Among all selenoproteins, SELENOW exhibits the highest expression level in the brain; however, this expression profile is not consistent with the current understanding of its biological functions in the brain. SELENOI is another selenoprotein that has only recently begun to be studied and may perform certain physiological functions because of its expression in the brain.

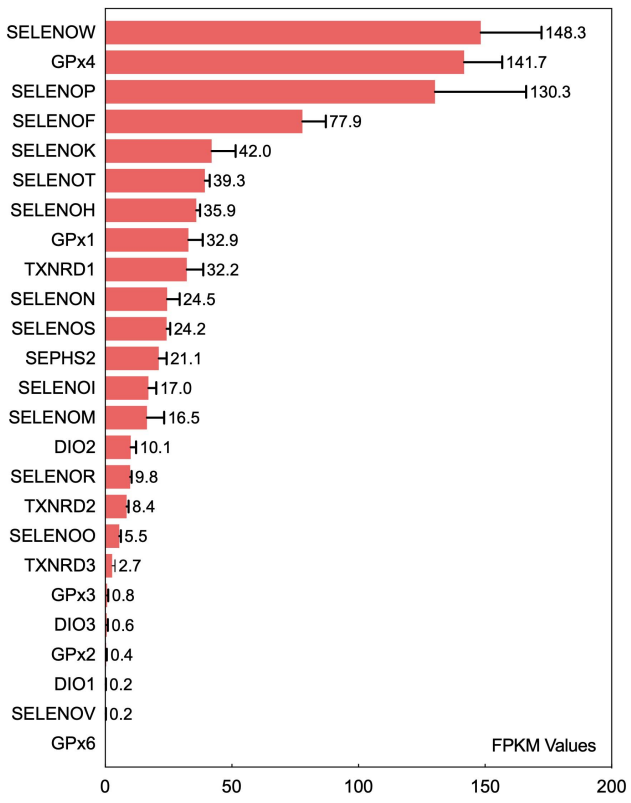

FIGURE 1 | Ranking of the mRNA expression levels of 25 selenoproteins in the human brain. The fragments per kilobase of exon model per million mapped reads (FPKM) values of 25 selenoproteins in the brain obtained from gene transcriptome data were analyzed to rank selenoproteins based on their expression level in the human brain. The data are from the literature (Fagerberg et al., 2014).

Furthermore, differences in the spatial expression levels of selenoproteins also determine their physiological functions in the brain (Zhang et al., 2008). The spatial expression profile data of selenoproteins in the human brain are incomplete. Zhang et al. (2008) thoroughly analyzed *in situ* hybridization data and the expression profiles of more than 210,000 genes in a genome-wide expression database of the brain of adult mice in the previously published Allen Brain Atlas (ABA) and showed that brain selenoproteins are enriched mainly in the hippocampus, olfactory bulb, neocortex, and cerebellar cortex; the expression levels of GPX4, SELENOW, and SELENOF are highest in these four regions (Zhang et al., 2008). Except for SELENOP, all selenoprotein genes with high expression levels in the brain have at least moderate expression levels in the hippocampal region. The oculomotor nucleus, Edinger-Westphal nucleus, nucleus raphe pontis, anteroventral periventricular nucleus, and dorsal premammillary nucleus have the lowest expression levels of selenoproteins, indicating that these regions might be less dependent on selenoproteins and Se (Zhang et al., 2008).

Selenoproteins and Brain Function

Brain and Neural Development

The importance of Se in brain development was confirmed in Selenop$^{-/-}$ mice (Schomburg et al., 2003). In a low-Se environment, newborn Selenop$^{-/-}$ mice exhibit severe hypoplasia and death (Hill et al., 2004; Schweizer et al., 2005). However, after overexpression of SELENOP in hepatocytes, the above neuropathological features were reversed when the mice were fed a Se-adequate diet (Renko et al., 2008). GPX4 and SELENOT are also indispensable for neural development in mice. GPX4 gene knockout induces embryonic lethality, and mice with conditional GPX4 knockout exhibit massive neurodegeneration before weaning (Seiler et al., 2008), which might be associated with selective loss of parvalbumin (PV) interneurons in mouse hippocampal and cortical regions (Wirth et al., 2010). Although GPX1 gene knockout does not affect the normal development of mice, GPX1 overexpression improves the differentiation of mouse embryonic stem cells into central neural stem cells, especially dopaminergic neurons (Gardaneh et al., 2011; Abasi et al., 2012). SELENOT is the only ER-resident selenoprotein whose knockout leads to embryonic lethality in mice. Neuron-specific SELENOT-knockout mice have significant decreases in the hippocampal, cortical, and cerebellar volumes due to neuronal apoptosis in the brain at postnatal day 7 (P7), leading to abnormal brain function in adult mice, which suggests a neuroprotective function of SELENOT in brain development (Castex et al., 2016).

Furthermore, SELENOW protects neurons against oxidative stress injury during neuronal development. H_2O_2-induced cell apoptosis is significantly increased in SELENOW-knockout primary neurons derived from the embryonic cerebral cortex (Chung et al., 2009). Recent studies of SELENOI (ethanolamine phosphotransferase 1, EPT1) showed that EPT1 mutation leads to cerebellar and brain stem atrophy, which can induce sensorineural deafness, blindness, and seizures. These results confirm the indispensable role of EPT1 in myelination and neurodevelopment and in the maintenance of phospholipid homeostasis in humans (Horibata et al., 2018). Although nervous system-specific deletion TXNRD2 does not affect the normal development of mice, cerebellar hypoplasia in TXNRD1-knockout mice occurs due to decreased proliferation of granule cell precursors within the external granular layer (EGL), indicating that TXNRD1 affects neuronal precursor cells and participates in the regulation of neuronal development (Soerensen et al., 2008). Thyroid hormone influences brain development through regulation of neuron and glial cell differentiation and myelin and synapse formation. DIO2 in astrocytes mediates the transformation of T4 into active T3, and DIO3 in neurons degrades T3 and T4 to stabilize thyroid hormone homeostasis in the brain (Bernal, 2000). Although the development and maturation of PV neurons in the cortex depend on thyroid hormone, no significant changes in these cells were observed in DIO2-knockout mice, and the most significant change was sensorineural hearing loss caused by retarded cochlear development (Ng et al., 2004, 2009). DIO3-knockout mice also exhibit developmental disorders in retinal receptors (Ng et al., 2010), indicating that DIOs can significantly affect the development of the visual and auditory systems in the brain.

Ca^{2+} Homeostasis and ER Stress

Seven ER-resident selenoproteins are involved mainly in regulating calcium flux, protein folding, and redox balance in the ER. Other selenoproteins are also involved in these physiological processes; for example, SELENOP responds to ER stress in hepatocytes (Zhao et al., 2016), and SELENOW regulates Ca^{2+} channels in muscle cells (Yao et al., 2016). However, according to current reports, only five ER-resident selenoproteins (SELENOK, SELENOM, SELENOS, SELENOT, and DIO2) participate in the regulation of ER homeostasis in the brain or neural cells. Previous studies have shown that the SELENOS-mediated complex, which is composed of SELENOK, valosin-containing protein (VCP) (an important ATPase on the ER membrane), Derlin (a chaperone protein), and an E3 ubiquitin ligase, transports misfolded proteins to the ubiquitin-proteasome system for degradation (Ye et al., 2004; Lee et al., 2015), indicating that SELENOS and SELENOK play important roles in protein folding and in the ER-associated degradation (ERAD) pathway. Neuronal SELENOS expression increases with ER stress (Curran et al., 2005; Gao et al., 2006), and SELENOS gene knockout results in ER stress-mediated apoptosis (Rueli et al., 2017). To date, no evidence indicates that SELENOS is directly involved in Ca^{2+} regulation. However, studies have shown that SELENOK can interact with a palmitoyltransferase (DHHC6) to affect Ca^{2+} flux through regulation of inositol 1, 4, 5-trisphosphate receptor (IP3R) palmitoylation (Verma et al., 2011; Fredericks et al., 2014) and that SELENOK overexpression increases IP3R-mediated free Ca^{2+} levels in microglia (Meng et al., 2019). Furthermore, a cyclic adenosine monophosphate (cAMP)-mediated increase in Ca^{2+} levels in neuronal cells was shown to significantly improve SELENOT expression. SELENOT overexpression also affects the basal Ca^{2+} level in cells, indicating that SELENOT regulates intracellular Ca^{2+} homeostasis (Grumolato et al., 2008). A study by Jo et al. (2019) in cell and animal models showed that mutation of Ala92 in DIO2 to Thr increased ER stress in different brain regions in mice and that the unfolded protein response (UPR) and hypothyroidism were present (Jo et al., 2019). Neuronal SELENOM overexpression has been shown to reduce H_2O_2-mediated intracellular Ca^{2+} flux. Conversely, SELENOM gene knockout promoted an increase in cytosolic Ca^{2+} levels, enhanced oxidative stress, and apoptosis (Reeves et al., 2010). In presenilin 2 (PS2)-overexpressing neurons, Ca^{2+} efflux from the ER was associated with a decrease in SELENOM expression (Hwang et al., 2005). However, the mechanism underlying the regulation of Ca^{2+} homeostasis by SELENOM remains unclear.

Synaptic Function and Neurotransmission

Because selenoproteins play diverse roles in development and maintain homeostasis in the CNS (Solovyev, 2015) selenoproteins may be assumed to play roles in signal transmission. Previous studies have shown that Se-mediated neurotransmission is active mainly in the dopaminergic system and that Se deficiency can induce chemical injury to dopaminergic terminals and neurons (Kim et al., 1999, 2000). However, few studies have confirmed the direct relationship between selenoproteins and neuronal signal transmission. SELENOP was the first selenoprotein identified to be associated with synaptic signal transmission. Alterations of synaptic transmission and long-term potentiation were observed in CA1 synapses of Selenop$^{-/-}$ mice (Peters et al., 2006). In addition, SELENOP interacts with the postsynaptic apolipoprotein E receptor-2 (ApoER2) which participates in reelin protein-mediated synaptic signal transmission (Weeber et al., 2002; Beffert et al., 2005). ApoER2 also forms a functional complex with the N-methyl-D-aspartate receptor (NMDAR) and localizes in the postsynaptic membrane of excitatory synapses (Krebs et al., 1991; Beffert et al., 2005). NMDAR is the major receptor for glutamate during neuronal synaptic transmission, and synaptic impairment is directly associated with NMDAR disorders in AD (Wang and Reddy, 2017). Notably, a recent study by Zhang et al. (2020) showed imbalanced levels of two functional subunits of NMDAR, namely, NMDAR2A and NMDAR2B, in the brains of SELENOK-knockout mice. Therefore, SELENOK may play a role in neuronal synaptic transmission, but further studies are needed to support this speculation. Furthermore, neuron-specific GPX4-knockout mice and Selenop$^{-/-}$ mice have spontaneous seizures since the corresponding regions (the cortical region in GPX4-knockout mice and the inferior colliculus in Selenop$^{-/-}$ mice) in the brain lack PV-expressing GABAergic interneurons (Wirth et al., 2010;

Pitts et al., 2012, 2015), suggesting that GPX4 and SELENOP participate in the process of GABAergic neuron transduction. Although SELENOR deficiency does not interfere with CNS development, the significantly downregulated expression levels of synaptic proteins and the synaptic receptor NMDAR can substantially affect the persistence of long-term potentiation and long-term depression (LTP/LTD) in the hippocampal CA1 region of the mouse brain (Shi et al., 2019). A study by Boukhzar et al. (2016) confirmed that SELENOT was necessary for dopamine production by dopaminergic neurons in PD model mice. During oxidative stress induced by neurotoxins, SELENOT regulates tyrosine hydrolase activity to increase dopamine levels, thus maintaining the functionality of dopaminergic neurons (Boukhzar et al., 2016).

Glial Cell-Mediated Neuroinflammation

Neuroinflammation in the brain is the excessive reaction of glial cells to pathological changes and usually presents as excessive activation and proliferation of microglia and astrocytes. The antioxidant capacity of GPX1 may participate in the regulation of the inflammatory cascade reaction in the brain. Gpx1$^{-/-}$ astrocytes reduce the H_2O_2 clearance rate to cause cell death (Liddell et al., 2006; Shin et al., 2018). However, mice with ischemic brain diseases and GPX1 overexpression have significantly fewer overactivated astrocytes and microglia than corresponding mice without GPX1 overexpression (Ishibashi et al., 2002). Functionally, GPX4 is the only GPX that can use phospholipid hydroperoxides as substrates (Brigelius-Flohe and Maiorino, 2013) and is the control center for lipid oxidation-mediated apoptosis. In a neuron-specific GPX4-knockout mouse model, stress-induced proliferation of astrocytes and neuroinflammation associated with neurodegenerative diseases occurred in the brain (Seiler et al., 2008). Interestingly, compared to GPX4 expression during perinatal brain development, it decreased in glial cells in the adult human brain; however, GPX4 expression in astrocytes was found to be significantly upregulated in a cerebral ischemia model (Savaskan et al., 2007). The same phenomenon was observed for SELENOS. Compared to its high expression in neurons, SELENOS expression is sparse in astrocytes. However, under pathological conditions or in the setting of brain injury, SELENOS expression increases significantly, especially in reactive astrocytes. A study by Fradejas et al. (2011) showed that SELENOS overexpression influenced astrocyte functions and reduced the secretion of proinflammatory cytokines to enhance resistance to ER stress and neuroinflammation. As mentioned in the previous sections, SELENOK promotes the migration and phagocytosis of microglia through the regulation of Ca^{2+} flux, and SELENOK is the only reported selenoprotein to have direct regulatory functions in microglia (Meng et al., 2019). SELENOM may reduce the oxidative stress level in cerebellar astrocytes, in addition to neurons, through the regulation of Ca^{2+} flux (Reeves et al., 2010). Furthermore, brains from SELENOP- and SELENOR-knockout mice exhibit obvious glial cell proliferation, suggesting that these two selenoproteins might also be involved in the regulation of neuroinflammation in the brain (Valentine et al., 2005).

Brain Function-Associated Selenoproteins and AD

The hippocampus, olfactory bulb, neocortex, and cerebellar cortex, which have high selenoprotein expression, are also vulnerable to neurodegenerative diseases; in particular, the hippocampus and cortex are major pathological regions in AD. A heat map of the expression levels of selenoproteins in 12 brain regions was plotted based on the *in situ* hybridization profile information in the ABA (**Figure 2**). This map shows that the high expression levels of the brain selenoproteins SEPHS2, SELENOK, SELENOR, DIO2, SELENOS, SELENOF, SELENOW, and SELENOT are even more pronounced in the hippocampal and cortical regions (the main pathological areas in the brain in AD) than in other brain regions, suggesting that these selenoproteins might be strongly associated with AD. Currently, research on selenoproteins in AD is limited. To further investigate the roles of selenoproteins in AD pathology, selenoproteins with the greatest research potential in AD are identified. Results in the literature were comprehensively assessed based on expression abundance in the brain, expression specificity in the hippocampal and cortical regions, and correlations of selenoproteins with four brain functions (closely associated with AD pathology). As shown in **Table 1**, selenoproteins were divided into 5 levels based on their expression levels (FPKM value: >100, 40–100, 15–40,

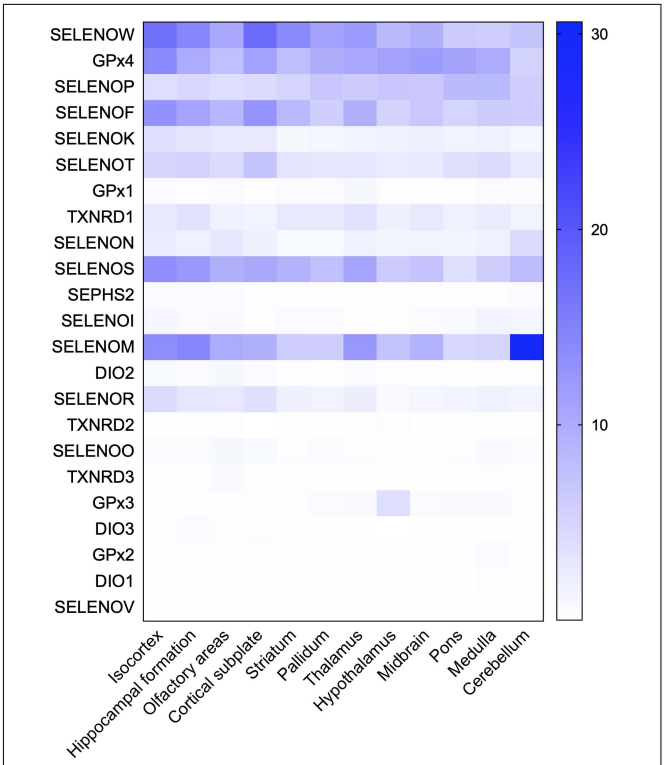

FIGURE 2 | Heat map of the distribution of 24 selenoproteins in the mouse brain. Based on genome-wide *in situ* hybridization data published in the ABA for the brains of adult mice, raw expression values of 23 selenoproteins in 12 regions in the mouse brain were analyzed and used to plot a heat map. The database contains no expression data for SELENOH in brain regions.

1–15, and <1) according to expression abundance in the brain. Selenoproteins were also divided into 5 levels based on their specific expression levels in the hippocampal cortex (percentage of expression in the hippocampal and cortical regions: >40, 30–40, 20–30, 10–20, and <10%), and divided into 2 levels based on the level of attention received in brain function studies (level 1: reported and level 2: massively reported), respectively. Based on the comprehensive coefficients (the total number of stars) obtained from the assessment, the top five selenoproteins are GPX4, SELENOP, SELENOK, SELENOT, and GPX1/SELENOM/SELENOS/SELENOW, which are consistent with the ranking of AD research correlation (the number of reports on each selenoprotein in AD-related research). Though this order may change with continued research on the roles of selenoproteins in brain function, these selenoproteins are hypothesized to be the most strongly associated with AD based on existing research data. Therefore, the relationships between these selenoproteins and AD are discussed further.

GPX4 and GPX1

Ferroptosis is a current research hotspot. Enhanced neuroinflammation and elevated lipid oxidation levels are hallmarks of pathological changes related to ferroptosis in the brain (Hambright et al., 2017). Recent studies have shown that lipid peroxidation-induced ferroptosis participates in neuronal death in AD (Stockwell et al., 2017). GPX4 reduces lipid peroxide production catalyzed by Fe^{2+}, and lipoxygenase (LOX) and is the key regulator of the ferroptosis pathway (Ingold et al., 2018; Wu et al., 2018). Significant iron accumulation and lipid peroxidation combined with reductions in glutathione (GSH) and GPX4 were observed in the hippocampus in AD patients (Yoo et al., 2010). In addition, GPX4 knockout in forebrain neurons of mice directly causes age-related neurodegenerative changes and obvious neuronal loss (Hambright et al., 2017), indicating that GPX4 is significantly associated with AD. Interestingly, the reactive oxygen species (ROS) level in GPX4-knockdown cells did not significantly change, but the level of lipid peroxidation products increased significantly. However, GPX1 does not decrease the lipid peroxide level mediated by GPX4 defects. These results suggest the specificity of GPX4 in protecting cells against lipid peroxidation damage (Yoo et al., 2010). Increased lipid peroxidation is considered an early event in AD. Iron accumulation promotes Aβ and tau aggregation (Yamamoto et al., 2002; Cheignon et al., 2018), whereas APP and tau collectively promote iron transport to cause the vicious

TABLE 1 | Analysis of 25 selenoproteins in AD studies.

Seleno-proteins	Expression abundance	Expression specificity	Brain function correlation				Comprehensive coefficient	AD research correlation
			Brain development	ER stress	Neuro-transmission	Neuro-inflammation		
GPX4	☆☆☆☆☆	☆☆☆	☆☆	—	☆	☆☆	13	6
SELENOP	☆☆☆☆☆	☆☆	☆☆	—	☆☆	☆	12	9
SELENOK	☆☆☆☆	☆☆☆☆	—	☆	☆	☆	11	1
SELENOT	☆☆☆	☆☆☆	☆☆	☆	☆	—	10	0
GPX1	☆☆☆	☆☆	☆☆	—	—	☆☆	9	5
SELENOM	☆☆☆	☆☆☆	—	☆☆	—	☆	9	4
SELENOS	☆☆☆	☆☆☆	—	☆☆	—	☆	9	2
SELENOW	☆☆☆☆☆	☆☆☆	☆	—	—	—	9	1
SELENOR	☆☆	☆☆☆☆	—	—	☆	☆	8	2
DIO2	☆☆	☆☆☆	☆	☆	—	—	7	1
SELENOF	☆☆☆☆	☆☆☆	—	—	—	—	7	0
TXNRD1	☆☆☆	☆☆☆	☆	—	—	—	7	0
SELENOI	☆☆☆	☆☆☆	☆	—	—	—	7	0
DIO3	☆	☆☆☆☆☆	☆	—	—	—	7	0
SEPHS2	☆☆☆	☆☆☆☆	—	—	—	—	7	0
SELENON	☆☆☆	☆☆☆	—	—	—	—	6	0
TXNRD2	☆☆	☆☆	☆	—	—	—	5	0
SELENOO	☆☆	☆☆	—	—	—	—	4	0
TXNRD3	☆☆	☆☆	—	—	—	—	4	0
DIO1	☆	☆☆☆	—	—	—	—	4	0
SELENOH	☆☆☆	—	—	—	—	—	3	0
GPX2	☆	☆☆	—	—	—	—	3	0
SELENOV	☆	☆☆	—	—	—	—	3	0
GPX3	☆	☆	—	—	—	—	2	0
GPX6	—	—	—	—	—	—	0	0

One star indicates 1 level, and the comprehensive coefficient is the total number of stars.

cycle of ferroptosis (Duce et al., 2010; Lei et al., 2017). In the brains of Gpx4$^{\pm}$ mice, the activity and protein level of β-secretase are significantly upregulated, and Aβ levels and amyloid plaque deposition are significantly increased (Chen et al., 2008). The above data indicate that the lipid peroxidation-mediated ferroptosis pathway regulated by GPX4 is involved in the neurodegenerative process in AD (**Figure 3D**), which may be a potential mechanism of Se on AD. Furthermore, as the downstream regulator of the ferroptosis pathway, the role of GPX4 in iron-mediated Aβ aggregation should be addressed, for the current data cannot confirm the direct effect of GPX4 on iron accumulation except for the inhibition of lipid peroxidation products.

Unlike for GPX4, the current research data on GPX1 cannot confirm its direct association with AD. Crack et al. (2006) used a GPX1-knockout primary neuron model and showed that depletion of GPX1 increased the sensitivity of neurons to Aβ toxicity. Similarly, the cognitive ability of

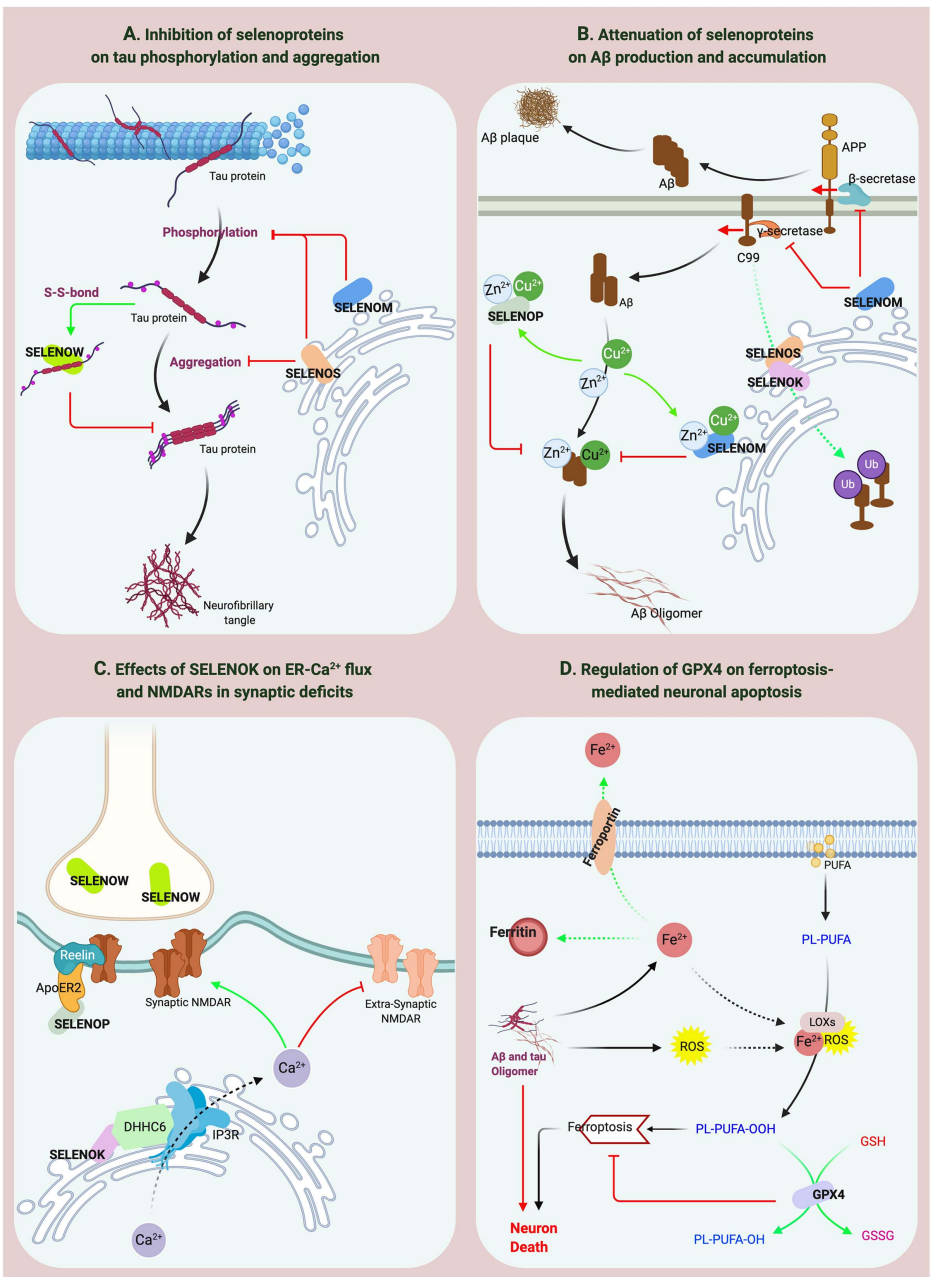

FIGURE 3 | The effects of selenoproteins on the pathological processes of AD. **(A,B)** SELENOP, SELENOW and SELENOM inhibit the aggregation of Aβ and tau. SELENOS participates in the pathological protein degradation process through ERAD. **(C)** SELENOK affects the distribution of synaptic receptors. **(D)** GPX4 regulates ferroptosis-mediated neuronal apoptosis.

GPX1-knockout mice treated with Aβ1-42 declined significantly, and the activity levels of the βII-isoform of protein kinase C (PKCβII) and extracellular signal−regulated kinase (ERK) in the brain significantly decreased. Re-expression of GPX1 in this mouse brain activated PKCβII-mediated ERK signal transduction to ameliorate Aβ1-42-induced memory impairment (Shin et al., 2020). In addition, studies of GPX4 and GPX1 gene polymorphisms show that certain GPX1 (rs1050450) and GPX4 (rs713041) genotypes are significantly associated with AD patients in a South Brazilian population (da Rocha et al., 2018). In addition, the Pro198Leu polymorphism in GPX1 has been reported to be associated with GPX1 enzyme activity. Recent studies found that this polymorphism can significantly affect the plasma Se level in AD patients, suggesting that the GPX1 genotype might impact the effect of Se supplementation in AD (Cardoso et al., 2012). Notably, in a survey of the Ecuadorian population, the Leu allele of GPX1 was a potential risk factor for AD (Paz-Y-Mino et al., 2010). However, no data are currently available to further validate whether the association between GPX1 polymorphisms and AD was due to the influence of GPX1 on Se levels in the body.

SELENOP and SELENOW

SELENOP is the most studied selenoprotein in AD-related research, possibly because of its high expression and multiple functions in the brain (Solovyev et al., 2018) and its association with the expression levels of many other selenoproteins. For example, loss of SELENOP can cause significant reductions in the brain expression levels of GPX4, SELENOK, SELENOM, and SELENOW (Hoffmann et al., 2007). Previous studies have reported that SELENOP expression increases in the brain with age (Lu et al., 2004) and that SELENOP gene expression is significantly increased in the brains of AD patients (Miller et al., 2008; Rueli et al., 2015). Bellinger et al. (2008) further confirmed that the spatial distributions of SELENOP, Aβ, and NFTs are colocalized in the brains of AD patients, suggesting that SELENOP is associated with AD. Ions of numerous metals, such as aluminum, zinc, copper, and iron, can promote Aβ aggregation during the pathological process of AD (Faller et al., 2014; Lei et al., 2020). Many Sec and Cys residues and a His-rich domain in SELENOP determine its metal-binding capacity (Turanov et al., 2015). SELENOP forms a coordination complex with Zn^{2+} and Aβ *in vitro* to inhibit Aβ aggregation and neurotoxicity (Du et al., 2013). SELENOP also interacts with the C-terminal domain of α-tubulin, which binds with tau protein to mediate the regulation of microtubule assembly (Du et al., 2014a), implying that SELENOP might be associated with the structure or function of tau protein. Studies by Du et al. (2014a) and Valentine et al. (2005) confirmed this hypothesis and showed that SELENOP significantly attenuates metalion-mediated tau aggregation and mitochondrial movement impairment and that SELENOP knockout induces structural and functional neuronal axon damage (Valentine et al., 2005; Du et al., 2014b). Furthermore, the antioxidant function of SELENOP also protects neurons against Aβ-induced toxicity. Interestingly, overexpression of Sec-deficient and histidine-rich SELENOP can inhibit the aggregation of Aβ and tau through the regulation of tropomyosin receptor kinase B (TrkB) signal transduction and Zn^{2+} homeostasis (Yue et al., 2020), significantly improving the learning and memory abilities of AD mice. These results indicate that the structural characteristics of SELENOP determine its potential active role in AD progression (**Figure 3B**). However, as Solovyev (2020) noted, although results clearly show that SELENOP is associated with AD, most relevant evidence is from *in vitro* studies. Therefore, more animal model and human-based studies are needed to obtain reliable conclusions (Solovyev, 2020). Moreover, compared with the inhibition of SELENOP on the aggregation of Aβ and tau, the interaction between SELENOP and ApoER2 and their effects on synaptic signal transmission deserve more attention as **Figure 3C** showed, which may be involved in synaptic dysfunction in AD.

Although dietary Se deficiency affects GPX activity in the brain, it does not reduce the SELENOW level (Sun et al., 2001). However, SELENOW in the brain is one of the selenoproteins whose expression is more affected by SELENOP (Hoffmann et al., 2007). Similar to most selenoproteins, SELENOW, as a GSH-dependent antioxidant, is involved in redox reactions (Jeong et al., 2002). SELENOW protects developing myoblasts against oxidative stress and inhibits interactions between 14-3-3 proteins and transcriptional activators to participate in muscle growth and differentiation (Loflin et al., 2006; Jeon et al., 2014). However, relatively few reports have addressed the role of SELENOW in brain function. SELENOW is highly expressed in the cerebral cortex, dentate gyrus, and hippocampus of postpartum rats and in the brain and spinal cord of developing embryos (Jeong et al., 2004; Chung et al., 2009). In addition, SELENOW is extensively expressed in synapses, and SELENOW expression is significantly decreased in the synaptosomes of SELENOP-knockout mice (Raman et al., 2013), suggesting that SELENOW has biological functions in neuronal synapses. A recent study by Chen et al. (2018) found that Cys37 of SELENOW and Cys322 of tau form a disulfide bond to inhibit tau protein aggregation, indicating that SELENOW may affect tau pathology and may be associated with AD (**Figure 3A**). However, the specificity of the disulfide bond between SELENOW and tau should be strictly evaluated. Based on the current data, the formation of a disulfide bond with tau at this site may be a general effect inhibiting tau protein aggregation, and further *in vivo* studies are needed to obtain more conclusive results.

SELENOK, SELENOT, SELENOS, and SELENOM

Our assessment indicates that ER-resident selenoproteins (SELENOK, SELENOT, SELENOM, and SELENOS), are strongly associated with AD, especially SELENOK, which has the strongest correlation due to its high expression in the brain and its specific expression in the cortex and hippocampus. Compared to other selenoproteins, SELENOK is highly expressed in immune cells (Verma et al., 2011). In addition, a Src-homology 3 (SH3) domain in SELENOK mediates its interaction with DHHC6, an enzyme that is also localized in the ER (Fredericks et al., 2014). The interaction between SELENOK and DHHC6 effectively catalyzes the palmitoylation of proteins such as IP3R and CD36 to stabilize their expression and further promote Ca^{2+} flux in the ER to activate immune cells (Fredericks et al., 2014;

Fredericks and Hoffmann, 2015; Marciel and Hoffmann, 2019). Microglia-mediated neuroinflammation is considered a major AD-inducing factor (Tejera and Heneka, 2016; Hansen et al., 2018), and SELENOK overexpression significantly increases the Ca^{2+} level and IP3R expression and promotes the migration and phagocytosis of microglia to regulate neuroimmunity and neuroinflammation in the brain (Meng et al., 2019). However, the direct relationship between SELENOK and AD has not been reported until recently. A study by Zhang et al. (2020) showed that SELENOK expression is significantly decreased in the brains of AD patients and mice and that SELENOK knockout is associated with pathological changes, such as intracellular Ca^{2+} flux dysregulation in neurons and an imbalance in the distribution of synaptic receptors, that are highly consistent with AD pathology. Currently, neuronal excitotoxicity mediated by the disequilibrium between synaptic and extrasynaptic NMDAR is a widely accepted pathogenic factor for synaptic loss in AD (Hardingham and Bading, 2010; Talantova et al., 2013; Huang et al., 2017). As **Figure 3C** shows, SELENOK participates in the regulation of ER-Ca^{2+} flux and the balance between synaptic and extrasynaptic NMDAR expression to restore synaptic deficits in AD. In addition, the effects of SELENOK on immune regulation and microglia-mediated neuroinflammation in the brain should also be addressed, and further exploration of the underlying mechanisms may reveal the role of SELENOK in AD pathology.

Because of its effect on Ca^{2+} flux in neural cells and dopaminergic neurotransmission, SELENOT may be associated with AD. To date, however, no research has been conducted regarding SELENOT in AD. SELENOT expression is significantly increased in the peripheral blood mononuclear cells and brain striatal tissue of PD patients. Both silencing and overexpression of SELENOT influence oxidative stress and apoptosis in dopaminergic neurons (Boukhzar et al., 2016; Shao et al., 2019; Zhang et al., 2019). Dopaminergic neurons are also closely associated with AD pathology, and several alterations in the dopaminergic system have been reported in AD patients (Burns et al., 2005; Rossato et al., 2009). Dopaminergic neurons in the prefrontal cortex participate in the formation of cognitive memory (Perkovic et al., 2018). Loss of dopaminergic neurons affects synaptic plasticity in hippocampal CA1 neurons in AD (Nobili et al., 2017). Although the functions of dopaminergic neurons differ across brain regions, the regulatory function of SELENOT in dopaminergic neurons in AD-related brain regions warrants in-depth study.

Cleavage of APP by β-secretase under pathological conditions produces a 99-amino acid C-terminal transmembrane fragment of APP (C99), which is further cleaved into Aβ. Accumulating evidence indicates that a high C99 level is the determining factor of AD (Lee et al., 2006; Lauritzen et al., 2012; Pera et al., 2017). C99 is a miscleaved protein; therefore, the ERAD pathway is activated in cells for its degradation (Bustamante et al., 2013). As mentioned in the previous sections, binding of SELENOS to chaperone proteins such as SELENOK and Derlin can mediate the UPR and ERAD to maintain ER homeostasis. Recently, Jang et al. (2017) showed that ubiquitination-dependent C99 degradation was inhibited and that the Aβ1-42 level was significantly increased in a SELENOS-knockdown cell model of AD, indicating that SELENOS participates in the C99 degradation process through ERAD. Interestingly, there is no obvious relationship between the spatial localization of SELENOS and Aβ in the brains of AD patients. However, SELENOS is expressed at high levels in neurons of NFTs (Rueli et al., 2017). Further studies have shown that the inhibition of SELENOS expression under ER stress increases tau phosphorylation and phosphorylated tau aggregation. Although SELENOS is involved in the production of Aβ and the hyperphosphorylation and aggregation of tau protein (as shown in **Figures 3A,B**), the relationships between SELENOS expression and AD pathology proteins are still rather confusing, especially in mouse model studies. In addition, due to limited research on the effects of Se supplementation on SELENOS levels and enhanced SELENOS expression on cognitive ability, the mechanism of Se in AD from the perspective of SELENOS has yet to be clarified.

Previous studies have shown that the transcription of SELENOM is significantly inhibited in the brains of familial AD transgenic mice overexpressing a human mutant presenilin 2 (PS2) gene, which disrupt Ca^{2+} homeostasis through promoting Ca^{2+} shuttling from the ER to mitochondria (Hwang et al., 2005). Later, a study by Yim et al. (2009) confirmed that SELENOM participates in APP cleavage and tau hyperphosphorylation, that the activity of α/γ-secretases in SELENOM-overexpressing mice changed after Se treatment, and that the phosphorylation of tau protein at multiple sites was inhibited through ERK pathway activation. Unfortunately, these results have not been verified in AD models. Mutation of the Sec residue in SELENOM to Cys revealed that SELENOM can bind to transition metal ions via its His-rich domain and thus regulate Zn^{2+}-mediated Aβ aggregation and neurotoxicity (Du et al., 2013). In an Aβ-expressing cell model, both full-length and truncated SELENOM were found to attenuate oxidative stress-induced mitochondrial damage through inhibition of Aβ oligomer formation (Chen et al., 2013). Still, the above anti-Aβ aggregation functions and mechanisms of SELENOM need to be further confirmed by *in vivo* studies, especially the effect and mechanism on homeostasis of Ca^{2+} and energy in the brain.

CONCLUSION

As selenoproteins are the representatives of Se performing its physiological functions, investigation of the functions of selenoproteins in the brain and the association of selenoproteins with AD pathology might be critical for elucidating the mechanism of action of Se. Selenoproteins (GPX4, SELENOP, SELENOK, SELENOT, GPX1, SELENOM, SELENOS, and SELENOW), which are highly expressed in the brain, specifically expressed in AD pathological regions, and closely associated with brain function, may be the most promising targets in AD research. Existing reports show that these selenoproteins may participate in pathological processes of AD, including neuronal apoptosis, pathological protein aggregation and clearance, synaptic dysfunction, and glial cell-mediated neuroinflammation (**Figure 3**). Although the regulatory functions and molecular

mechanisms of the above selenoproteins require further validation and exploration, this review provides relatively sufficient and reliable research data and directions for future studies on Se and AD.

AUTHOR CONTRIBUTIONS

Z-HZ: makes substantial contributions to conception and design, and literature research and annalysis of data, and the manuscript preparation and editing. Z-HZ and G-LS: participate in revising the manuscript critically for important intellectual content, and give final approval of the version to be submitted and any revised version. Both authors contributed to the article and approved the submitted version.

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Selenoprotein P Modulates Methamphetamine Enhancement of Vesicular Dopamine Release in Mouse Nucleus Accumbens Via Dopamine D2 Receptors

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Dopamine (DA) transmission plays a critical role in processing rewarding and pleasurable stimuli. Increased synaptic DA release in the nucleus accumbens (NAc) is a central component of the physiological effects of drugs of abuse. The essential trace element selenium mitigates methamphetamine-induced neurotoxicity. Selenium can also alter DA production and turnover. However, studies have not directly addressed the role of selenium in DA neurotransmission. Selenoprotein P (SELENOP1) requires selenium for synthesis and transports selenium to the brain, in addition to performing other functions. We investigated whether SELENOP1 directly impacts (1) DA signaling and (2) the dopaminergic response to methamphetamine. We used fast-scan cyclic voltammetry to investigate DA transmission and the response to methamphetamine in NAc slices from C57/BL6J SELENOP1 KO mice. Recordings from SELENOP1 KO mouse slices revealed reduced levels of evoked DA release and slower DA uptake rates. Methamphetamine caused a dramatic increase in vesicular DA release in SELENOP1 KO mice not observed in wild-type controls. This elevated response was attenuated by SELENOP1 application through a selenium-independent mechanism involving SELENOP1-apolipoprotein E receptor 2 (ApoER2) interaction to promote dopamine D2 receptor (D2R) function. In wild-type mice, increased vesicular DA release in response to methamphetamine was revealed by blocking D2R activation, indicating that the receptor suppresses the methamphetamine-induced vesicular increase. Our data provide evidence of a direct physiological role for SELENOP1 in the dopaminergic response to methamphetamine and suggest a signaling role for the protein in DA transmission.

Keywords: selenoprotein P, apolipoprotein E receptor 2, methamphetamine, dopamine, fast-scan cyclic voltammetry

INTRODUCTION

The mesolimbic system facilitates the rewarding effects of stimuli such as food, social interaction, and drugs of abuse (Nestler and Carlezon, 2006). Central to this function is the release of the neurotransmitter dopamine (DA) in the nucleus accumbens (NAc) in the ventral striatum, from afferents originating in the midbrain ventral tegmental area. Mesolimbic DA transmission is an essential causative factor in addiction (Wise, 1998; Koob and Le Moal, 2001). Methamphetamine is an illicit and highly addictive psychostimulant that is a type of amphetamine, a class of drugs that potentiate dopaminergic transmission. Amphetamines inhibit DA uptake through the DA transporter (DAT), resulting in elevated levels of extracellular DA in the synapse (Seiden et al., 1993; Sulzer, 2011). They are also capable of entering DA terminals and inducing the release of DA from vesicles into the cytosol by disrupting vesicular monoamine transporter-2 (VMAT-2) function. The increased cytoplasmic DA results in reverse transport of DA through DAT, a phenomenon known as "DA efflux" (Hedges et al., 2018). Daberkow et al. (2013) reported that D-amphetamine also causes an increase in vesicular DA release, while other studies have disputed this finding (Siciliano et al., 2014). Excessive methamphetamine exposure is neurotoxic, primarily causing deterioration of dopaminergic terminals, and chronic use causes cognitive deficits (Seiden et al., 1988; Volkow et al., 2001; Johanson et al., 2006).

Selenium, an essential trace element, is required for proper brain function (Pillai et al., 2014). Proteins of the selenoprotein family incorporate the trace element to form selenocysteine (Sec), the 21st amino acid (Bellinger et al., 2009). Selenoproteins serve a variety of roles, most notably as antioxidants, and production is highly dependent on dietary selenium availability (Ogawa-Wong et al., 2016). Previous studies indicated an interaction between selenium and the DA system (Castano et al., 1993, 1995, 1997; Rasekh et al., 1997; Romero-Ramos et al., 2000). Selenium supplementation protects against methamphetamine-induced neurotoxicity in rodent and *in vitro* models (Imam et al., 1999; Kim et al., 1999), whereas selenium deficiency potentiates toxicity (Barayuga et al., 2013). Dietary selenium restriction lowers selenoprotein expression levels and can increase the turnover of DA and its metabolites in rodent striatum, as measured by *in vivo* microdialysis (Romero-Ramos et al., 2000). It is unclear, however, how selenium affects DA transmission and what function selenoproteins may have in DA release.

Selenoprotein P (SELENOP1) is a secreted glycoprotein produced primarily in the liver, and in lesser amounts in other tissue. SELENOP1 is unique among selenoproteins in that it contains 10 Sec residues instead of only one (Burk and Hill, 2009). SELENOP1 is primarily considered a selenium transporter that travels through the blood stream delivering selenium to different body regions including the brain. Genetic deletion of SELENOP1 decreases brain selenium content by roughly 50%, similar to the effects of long-term dietary selenium restriction (Nakayama et al., 2007). Dietary supplementation with excess selenium can restore brain selenium levels in SELENOP1 knockout (KO) mice through non-SELENOP1 mechanisms (Burk and Hill, 2015) and prevent most resulting neurological impairments (Hill et al., 2003; Schomburg et al., 2003; Nakayama et al., 2007). Therefore, we investigated SELENOP1 KO mice to determine how restricted selenium delivery to the brain influences dopaminergic transmission and responses to methamphetamine. We used fast-scan cyclic voltammetry (FSCV) to measure DA release and re-uptake events in mouse NAc brain slices (Yorgason et al., 2011).

This study provides the first evidence, to our knowledge, that a specific selenoprotein directly modulates DA transmission. Our findings demonstrate SELENOP1 signaling via apolipoprotein E receptor 2 (ApoER2) that is independent of selenium. This signaling limited DA release in the presence of methamphetamine, potentially contributing to the ability of selenium to protect against methamphetamine-induced neurotoxicity (Imam et al., 1999; Kim et al., 1999; Barayuga et al., 2013). Finally, our results complement previous reports that methamphetamine augments vesicular DA release in the striatum, a point of contention in amphetamine research (Daberkow et al., 2013; Siciliano et al., 2014).

MATERIALS AND METHODS

Animals

All mouse care and experimental procedures were approved by the UH Manoa Institutional Animal Care and Use Committee (UH Manoa IACUC), protocol number 10–742, and conducted in accordance with the National Research Council's *Guide for the Care and Use of Laboratory Animals* and the ARRIVE guidelines. We used SELENOP1 KO mice with a C57/BL6J background (Hill et al., 2003) initially obtained from Vanderbilt University, and WT C57/BL6J mice initially obtained from Jackson Laboratories. As homozygous male SELENOP1 KO mice are sterile (Hill et al., 2003), the strain was maintained by breeding with the C57/BL6J mice for breeders, and experiments used homozygous SELENOP1 KO offspring. When possible, homozygous WT littermates of SELENOP1 KO mice were utilized. All mice used were 3–5 months of age. Littermates were group-housed up to 5 in a cage on a light/dark cycle and allowed access to food and water *ad libitum*. Mice were fed standard lab chow (Envingo, Cat#2920X) containing 0.23 ppm selenium. For indicated experiments, SELENOP1 KO mice were supplemented with selenium by adding sodium selenite (1 mg/ml) to the drinking water following weaning. No other agents or conditions were utilized prior to tissue harvest for experiments. Studies utilized brain slices from both male and female mice. No significant or apparent sex differences were observed within wild-type (WT) or SELENOP1 KO groups in terms of basal measurements and methamphetamine response. Therefore, data from male and female mouse brain slices were combined within each comparison.

Brain Slice Preparation

Brain slices containing NAc were obtained from WT and SELENOP1 KO mice and FSCV employed to assess DA release and reuptake at under baseline conditions and in the presence of methamphetamine. Methamphetamine and other

pharmacological agents were applied to NAc slices via perfusion with artificial cerebral spinal fluid (ACSF) while monitoring changes in extracellular DA concentrations.

Mice were euthanized via rapid cervical dislocation to avoid effects of anesthetic remnants on neurophysiology. Mouse brains were removed and placed in ice-cold ACSF consisting of: 130.00 mM NaCl, 3.50 mM KCl, 10.00 mM glucose, 24.00 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.50 mM $MgSO_4$, 2.00 mM $CaCl_2$, and bubbled with carbogen gas (95% O_2/ 5% CO_2). Coronal brain slices of 350 μm containing NAc were obtained using a Leica VT 1200 S vibrating blade microtome. Hemispheres of striatal slices were separated using a scalpel and placed into a slice incubation chamber containing oxygenated ACSF. Following recovery at room temperature for 30 min, slices were transferred to a heated water bath at 33°C for at least 30 min prior to experimentation.

Voltammetric Recordings

For *ex vivo* FSCV experiments, brain slices were transferred to a slice recording chamber and constantly perfused with oxygenated ACSF at 33°C at a flow rate of 3 mL/minute. For recordings, a carbon fiber electrode (CFE) was placed ∼100 μm below the surface of the brain slice in the NAc shell under the guidance of a microscope with a 10× objective lens. The stimulating electrode was placed 100–200 μm from the tip of the CFE at the same depth of the CFE. Extracellular DA concentrations were measured using a Dagan CHEM-CLAMP voltage clamp amplifier. A command voltage (CV) was applied to the CFE and scanned linearly in a triangular waveform from −0.4 to 1.2 V at a rate of 400 V/second. The CV induces DA oxidation, resulting in a current conductance proportional to the concentration of extracellular DA present (**Figure 1A**).

For evoked DA release measurements, the CV was applied at a frequency of 10 Hz (every 0.1 s), and the resulting current response to each CV was measured to produce a cyclic voltammogram with a peak current response representing DA oxidation at its oxidation potential (∼0.6 V). A 1-min epochs were collected every 2 min coinciding with a single stimulation train. Cyclic voltammograms were regularly referenced to confirm the specificity of the current output to DA oxidation. Data were digitized using an NI-6221 analog-to-digital converter (National Instruments) and analyzed using Demon Voltammetry software (Yorgason et al., 2011). DA release was elicited via electrical stimulation, and the resulting signal analyzed to assess release and reuptake (**Figures 1B,C**). DA release was evoked using a 10-pulse train of 0.5 ms biphasic stimuli (370 μA) at 20 Hz every 2 min using an A365 Stimulus Isolator (World Precision Instruments) to simulate phasic DA release events (Ferris et al., 2013). In initial assessments, stimulation trains of 1-, 2-, and 10-pulses at 20 Hz were used to test the level of responsiveness to varying degrees of stimulation.

After observing 30 min of stable baseline responses (2-min epochs), methamphetamine in ACSF was applied via perfusion for 30 min, followed by washout with regular ACSF for another 30 min. In some experiments, chemicals were applied for at least 15 min prior to methamphetamine application and for total durations indicated in figures. Drugs and purified proteins were diluted in ACSF and delivered via perfusion during experiments. Methamphetamine was used at a working concentration of 10 μM (2 times the measured EC50 when applied to mouse NAc slices) (Hedges et al., 2018). Concentrations are indicated in the RESULTS sections for: Quinpirole (Sigma, Q102); Sulpiride (Sigma, S8010). Stock solutions were made up in Milli-Q water at a 10,000× concentration to minimize any potential effect on the osmolarity of ACSF chemical components.

Electrode Fabrication and Calibration

Carbon fiber electrode were produced by inserting a 7 μm diameter carbon fiber into a borosilicate glass capillary tube, OD: 1.2 mm, ID: 0.696 mm, L: 100 mm, (Hilgenberg) using negative air pressure. Carbon fiber-containing capillary tubes were then pulled on a David Kopf model 700B vertical pipette puller (David Kopf Instruments) and the protruding fiber cut to a length of 100 μm from the tip of the pipette, and sealed with a cyanoacrylate compound. CFEs were calibrated by perfusing the electrode in the recording chamber with ACSF containing 10 μM DAHCl (Sigma) and observing the maximum resultant current (nA) to produce a "current to DA concentration" conversion factor. CFEs were backfilled with 3 M KCl. Stimulating electrodes were pulled on a Sutter P-1000 Flaming/Brown micropipette puller (Sutter Instrument) using borosilicate glass capillary tubes, OD: 1.5 mm, ID: 0.86 mm, L: 100 mm, (Sutter Instrument) and the tips were broken to yield a 50 μm diameter opening. Stimulating electrodes were backfilled with ACSF.

Data Analysis

Changes in current amplitudes following stimulation relative to the currents 100 ms prior to stimulation were converted to relative DA concentrations using a conversion factor determined by calibrating each CFE to ACSF containing 10 μM DA·HCl (Sigma). The maximum concentration value observed post-stimulation was extracted from each epoch, plotted over time, and normalized to the average baseline recordings. Statistical comparisons were made using the maximum concentration value recorded for each experiment post-methamphetamine application. Comparisons were also made of the maximum percent increase reached over baseline.

Data were analyzed using a curve-fitting model in the Demon Voltammetry software that incorporates Michaelis-Menten kinetics to discern contributions of DA release and reuptake according to the following equation (Wightman et al., 1988; Wightman and Zimmerman, 1990; Yorgason et al., 2011):

$$\frac{d\,[\mathrm{DA}]}{dt} = \frac{f\,[\mathrm{DA}]\,\mathrm{p} - \mathrm{V_{max}}}{(\mathrm{K_m}/[\mathrm{DA}]) + 1}$$

Changes in the extracellular DA concentration [DA] were modeled as DA release in competition with DA reuptake (Wightman et al., 1988; Wightman and Zimmerman, 1990). The DA release per pulse, [DA]p, represents the concentration of DA released evoked by an individual electrical stimulation pulse p for a train of stimuli given at frequency *f*. The Michaelis-Menten constant Vmax represents the maximal rate of DA uptake resulting from DAT activity and correlates with the amount of DAT present. Km represents the apparent affinity of DA for

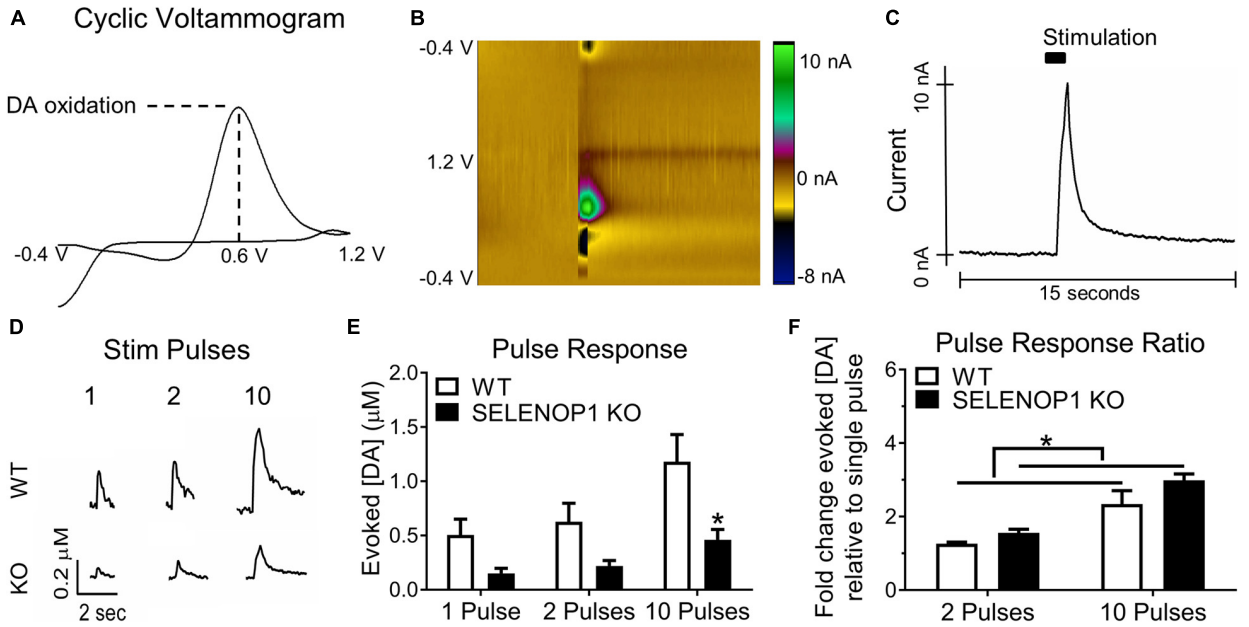

FIGURE 1 | Deletion of SELENOP1 resulted in reduced evoked DA responses measured in NAc brain slices. **(A)** sample cyclic voltammogram using 10-pulse stimulation showing peak oxidation potential around 0.6 V and used to confirm DA detection. **(B)** 3-dimensional heat map depicting the measured current according to color scale (right) for each point in voltage sweep (*y*-axis), plotted over time (*x*-axis). **(C)** sample evoked DA signal derived from the peak current of the plot in **(B)**, following stimulation at 5 s. **(D)** representative traces from C57 WT mice and C57 SELENOP1 KO mice, aged 3–5 months, in response to 1-, 2-, and 10-pulse stimulation. **(E)** mean (± SEM) peak DA responses from 1-, 2, and 10-pulse stimulation. Two-way ANOVA revealed a significant effect of genotype on the amount of DA released ($F_{(1,24)}$ = 17.38; p = 0.0003). A 1-pulse stimulation caused DA release from slices of WT mice (0.5 ± 0.15 µM; n = 5) and slices from SELENOP1 KO mice (0.15 ± 0.05 µM; n = 5), 2-pulse stimulation-induced DA release (0.63 ± 0.17 µM, n = 5 from WT mice compared to 0.22 ± 0.05 µM, n = 5 for SELENOP1 KO mice). A 10-pulse stimulation induced greater DA release in WT (1.2 ± 0.37 µM; n = 5) than in SELENOP1 KO mice (0.46 ± 0.12 µM; n = 5; *p = 0.0198, Tukey's multiple comparisons test). **(F)** mean (± SEM) ratios of either 2-pulse-elicited responses or 10-pulse-elicited responses to 1-pulse-elicited responses. Two-way ANOVA revealed a significant effect of genotype ($F_{(1,16)}$ = 4.962; *p = 0.0406). The ratio of 2-pulse to 1-pulse responses in SELENOP1 KO mice was 1.6 ± 0.1 µM; n = 5, and in WT mice it was 1.26 ± 0.04 µM; n = 5. The ratio of 10-pulse to 1-pulse responses was comparable between SELENOP1 KO and WT mice (2.9 ± 0.3 µM; n = 5 and 2.3 ± 0.1 µM; n = 5, respectively). All values reported are mean ± SEM.

DAT and is used as an approximation of the degree of DAT inhibition observed (Yorgason et al., 2011). In short trains of successive stimuli, the DA released with each subsequent pulse may vary from short-term release plasticity (Sulzer et al., 2016). We therefore refer to the total released DA ([DA]r) to represent the sum of the individual [DA]p for each pulse within a phasic-like stimulus train. Total vesicular DA release was, therefore, calculated as $[DA]\,r = \int_{p=1}^{n} [DA]\,p$, where "$n$" = the number of stimulus pulses per train (10 pulses for all experiments, unless otherwise indicated). For baseline recordings, models used a Km value of 160 nM in accordance with previous studies on the affinity of DA for DAT in rodent striatum (Wu et al., 2001). Vmax was measured at baseline and kept constant in models for the duration of experiments. The apparent Km was adjusted to the best fit for changes in DA signal decay exhibited upon methamphetamine application, in addition to any potential effects on DA uptake rates by other agents applied to the brain slices. Although methamphetamines may affect the trafficking and surface expression of DAT, it remains difficult to dissociate whether differences in DA release are due to changes in Vmax from or Km, as reported by other studies (Ramsson et al., 2011). Additionally, previous voltammetric analysis of the effects of amphetamine on brain slices did not reveal a change in Vmax (Jones et al., 1999). Nonetheless, changes in Vmax caused by methamphetamine cannot be ruled out in the current study and, thus, represents a caveat to the analysis presented herein.

SELENOP1 Protein Purification

SELENOP1 protein, including mutants, was purified from WT C57/BL6 mouse serum with an antibody affinity column using a previously described protocol (Kurokawa et al., 2014). Monoclonal SELENOP1 antibody (9S4, RRID:AB_2617215) was coupled to AminoLink Plus Coupling Resin (Pierce) and applied to a 10 mL serological pipette. Serum was first diluted 1:2 in chilled PBS and centrifuged at 14,000 *g* for 10 min at 4°C and the supernatant containing protein collected. The supernatant was run through the column and followed by a brief wash with PBS. A wash of 1 M NaCl was then applied to the column, followed by PBS. A 50 mM glycine pH 2.5 was then run through the column to elute SELENOP1 from its bound state, and the eluate collected in 1 mL fractions in tubes containing 1 M Tris pH 8.0. Fractions were tested for protein content by adding 5 µL of eluate to 10 µL drops of Bradford Assay Reagent. After all fractions were collected, the column was rinsed with PBS until wash out reached a pH of at least 7.4. The fractions from each elution that contained the highest protein

concentration were concentrated to 1 mL of stock protein of 3.6 μM using a Vivaspin Centrifugal Concentrator (Sartorius). SELENOP1 mutations were previously described in Kurokawa et al. (2014). The full-length all-Cys mutant is a full-length SELENOP1 peptide with all Sec residues mutated to Cys residues. The N-terminal fragment mutant is an all-Cys SELENOP1 N-terminal peptide lacking the C-terminal region. The Δ234-237 mutant is a full-length all-Cys SELENOP1 peptide with an essential region of the ApoER2 binding domain deleted. Thus, it is unable to bind ApoER2 as previously demonstrated (Kurokawa et al., 2014).

Statistical Analysis

Statistical comparisons were made using the peak concentration signal recorded post-methamphetamine application. For each type of experiment, an "n" specifies data of a single brain slice taken from one animal. For each animal used, only one slice was used for a given type of experiment, and additional slices from the same animal were used for different types of experiments when possible. This exploratory study was not preregistered. Analysis of DA measurements and model fitting from recorded data were performed blind to genotype and experimental conditions, although data recording was not. Animals were not randomized, and included 22 WT and 22 KO mice. Data was not included in analysis if the baseline DA responses were less than 6 nA for evoked DA release or varied by more than 10% during baseline recordings of non-stimulated DA changes. One-way ANOVA with Tukey's multiple comparisons test used for between-subject group comparisons between multiple groups with a single variable, and two-way ANOVA with Dunnett's multiple comparisons test used for between-subject comparisons with multiple groups and/or more than one variable. Otherwise, unpaired *t*-test was used to compare sets of two groups. The following criteria were used for significance: at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). All statistical analysis was executed in GraphPad Prism 6 software (GraphPad Software, Inc.). All data are represented as mean ± SEM.

RESULTS

DA Release Is Reduced in SELENOP1 KO Mice

Changes in extracellular DA concentration were measured in NAc slices via FSCV (**Figures 1A–C**). To determine if genetic deletion of SELENOP1 affects DA release we stimulated NAc slices with either single pulses, 2-pulse trains, or 10-pulse trains at 20 Hz. Sample traces are shown in **Figure 1D**. SELENOP1 KO slices released less DA than WT slices in response to multiple stimulation profiles (two-way ANOVA; $F_{(1,24)} = 17.38$, $p < 0.001$). Single pulse and 2-pulse stimulation similarly evoked less DA release in SELENOP1 KO slices. A 10-pulse stimulation elicited significantly greater DA release in WT than in SELENOP1 KO slices (Tukey's; $p < 0.05$) (**Figure 1E**). The mean early slope, representing the presumed release portion of evoked signals was also lower in baseline measurements from SELENOP1 KO slices (unpaired *t*-test; $t_{(8)} = 4.364$, $p < 0.005$) (**Supplementary Figures 1A–C**). To probe for potential differences in release probability (Cragg, 2003; Condon et al., 2019), we compared the fold change in DA release in response to 2- and 10-pulse trains relative to a single pulse within each genotype. *Post hoc* analysis following two-way ANOVA did not reveal a statistically significant difference between genotypes in terms of the 2-pulse or 10-pulse response ratio. The 10-pulse to the single pulse response ratios were roughly three-fold higher for both SELENOP1 KO and WT slices. There was a significant effect of genotype on both ratios, however, with greater values detected in SELENOP1 KO mouse slices (**Figure 1F**).

SELENOP1 KO Mice Exhibit Enhanced Vesicular DA Release in Response to Methamphetamine

We next examined whether SELENOP1 KO mice have an altered response to methamphetamine. SELENOP1 KO slices and WT slices both exhibited an immediate increase in the evoked DA response post-methamphetamine application (**Figures 2A,B**). Although the max post-stimulation extracellular DA concentration observed in SELENOP1 KO slices in the presence of methamphetamine was smaller compared to WT slices ($t_{(12)} = 2.293$, $p < 0.05$) (**Figure 2B**), the percent increase from baseline was nearly double that of WT slices ($t_{(12)} = 4.82$, $p < 0.001$) (**Figure 2C**). These evoked DA signals gradually decayed toward baseline levels, although decay was slower in SELENOP1 KO slices.

The curve-fitting analytical model in the Demon Voltammetry software simulates Michaelis-Menten kinetics to resolve the release and uptake components of the evoked DA signal, which are occurring simultaneously and in opposition to each other for the duration of the signal (**Supplementary Figure 2A**; Yorgason et al., 2011). We used this modeling system to estimate the magnitude of total vesicular DA release, [DA]r. Consistent with the observed reductions in peak extracellular DA concentration and reduction in the rising slope of baseline signals, SELENOP1 KO slices also exhibited lower [DA]r at baseline compared to WT slices ($t_{(27)} = 4.188$, $p < 0.001$) (**Supplementary Figure 1D**). In response to methamphetamine, SELENOP1 KO slices exhibited a robust initial increase in [DA]r that gradually decreased in amplitude toward baseline with successive stimulations (**Figure 2D** and **Supplementary Figure 2B**). In contrast, WT slices displayed only a slight increase that quickly dropped below baseline. The averaged max percent increase over baseline in [DA]r was greater in the SELENOP1 KO slices than in WT controls ($t_{(12)} = 5.481$, $p < 0.0001$). In order to observe DA efflux caused by methamphetamine, separate experiments were conducted during which changes in extracellular DA concentration were monitored in the absence of stimulation before and during methamphetamine application. There was no significant difference in the peak non-stimulated response to methamphetamine detected between WT (10.6 ± 2.3 μM) and SELENOP1 KO (6.9 ± 1.1 μM) mice (student's *t*-test: $p = 0.25$; data not shown).

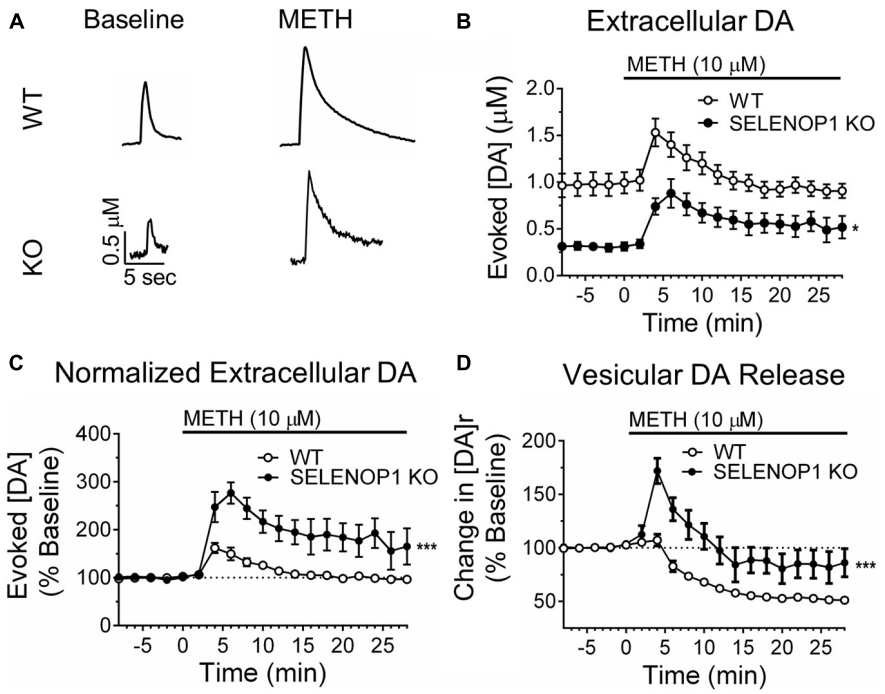

FIGURE 2 | Methamphetamine enhanced vesicular DA release in SELENOP1 KO mice. **(A)** representative traces of evoked DA signals from C57 WT mice and C57 SELENOP1 KO mice before and after 10 μM methamphetamine (METH). **(B)** time course of DA release evoked in slices from WT and SELENOP1 KO mice with stimulus trains every 2 min. Peak evoked extracellular DA concentrations following exposure to METH was lower in SELENOP1 KO slices (0.9 ± 0.1 μM; $n = 8$) than in WT control slices (1.5 ± 0.1 μM; $n = 6$) (*$p = 0.0127$). **(C)** time course of evoked DA release represented as a percent change over baseline. Methamphetamine increased DA release in SELENOP1 KO NAc (292.9 ± 27.1%; $n = 6$) significantly more than in C57 WT NAc (163.1 ± 11.8%; $n = 8$; ***$p = 0.0004$) relative to baseline levels. **(D)** time course of vesicular DA release in response to methamphetamine as a percent change over baseline. Vesicular DA release is represented by the variable [DA]r, the total concentration of DA released per stimulation train. Methamphetamine induced a slight increase in [DA]r in WT mice that subsequently dropped below baseline. SELENOP1 KO mice exhibited a substantially greater increase in [DA]r upon methamphetamine application (171.9 ± 1.86%; $n = 6$) compared to WT mice (112.9 ± 3.14%; $n = 8$; ***$p = 0.0001$). All values reported are mean ± SEM.

DA Uptake Is Impaired in SELENOP1 KO Mice

We used Michaelis-Menten kinetic modeling to depict changes in DA uptake in SELENOP1 KO mice. Vmax represents the maximal rate of DA uptake when available DAT are saturated with DA. The initial decay of the evoked DA signal following DA release is primarily controlled by Vmax (**Supplementary Figure 2A**). Vmax was reduced in SELENOP1 KO slices, indicating slower basal DA uptake rates compared to WT slices ($t_{(44)} = 7.021$, $p < 0.0001$) (**Supplementary Figure 2C**).

Methamphetamine inhibition of DAT was calculated as the Michaelis-Menten constant Km, representing the apparent affinity of DA for DAT. The latter portion of the DA signal decay is taken to be largely a function of the apparent Km (**Supplementary Figure 2A**). Methamphetamine elicited comparable increases in apparent Km in both WT and SELENOP1 KO slices indicating similar levels of DA uptake inhibition (**Supplementary Figure 2D**).

Several studies have shown that gender can impact phenotypic differences in SELENOP1 KO mice that can be mitigated by selenium supplementation (Hill et al., 2003, 2004; Valentine et al., 2005; Raman et al., 2012). However, we did not find any difference between male and female mice within each genotype for the differences in DA release and reuptake kinetics reported above (**Supplementary Figures 3A–I**).

SELENOP1 KO Mice Have Elevated Expression of VMAT-2 and D2R

To determine potential changes in the SELENOP1 KO mice related to changes in DA release, we measured changes in protein levels in brain lysates from WT and SELENOP1 KO mice. Expression of VMAT-2 ($t_{(5)} = 5.007$, $p < 0.01$) and dopamine D2 receptor (D2R) ($t_{(5)} = 7.268$, $p < 0.001$) were both elevated in SELENOP1 KO ventral midbrain (**Supplementary Figure 4A**). No changes in TH expression or DAT expression were detected in ventral midbrain (**Supplementary Figure 4A**). No significant difference in TH expression was observed between WT and SELENOP1 KO mice in the ventral striatum, despite observing smaller electrically evoked DA signals in SELENOP1 KO slices (**Supplementary Figure 4B**). VMAT-2 expression was increased in SELENOP1 KO ventral striatum ($t_{(5)} = 3.300$, $p < 0.05$), further suggesting increased vesicular packaging of DA in SELENOP1 KO mice (**Supplementary Figure 4B**). In order to preliminarily probe for changes in the amount of vesicular DA packaging per DA terminal we compared the expression

of VMAT-2 to DAT for each subject. The ratio of VMAT-2 expression to DAT expression was significantly increased in SELENOP1 KO ventral striatum ($t_{(4)} = 3.248$, $p < 0.05$; **Supplementary Figures 4C,D**).

SELENOP1 Protein Can Prevent the Methamphetamine-Induced Increase in Vesicular DA Release

SELENOP1 deletion decreases brain selenium content (Hill et al., 2003). Decreased selenium availability could, in turn, contribute to our findings, potentially via reduced expression of other members of the selenoprotein family. Previous studies demonstrated that dietary selenium supplementation can reverse many neurological deficits of SELENOP KO mice (Hill et al., 2003; Schomburg et al., 2003; Nakayama et al., 2007). To test whether reduced selenium availability caused or contributed to our results, we supplemented the drinking water of SELENOP1 KO mice with selenium (1 mg/mL) immediately post-weaning to partially restore brain selenium (Hill et al., 2003; Schomburg et al., 2003; Nakayama et al., 2007). Selenium supplementation in SELENOP1 KO mice did not significantly alter baseline DA release or the peak methamphetamine response relative to non-supplemented SELENOP1 KO mice (**Supplementary Figures 5A–C**). Moreover, selenium supplementation did not restore baseline evoked DA signals in SELENOP1 KO NAc, nor did it affect the measurement of Vmax or Km (**Supplementary Figures 5D,E**). These findings argue against the possibility that the increased [DA]r and other changes in the KO mice relative to WT animals are due to an overall reduction in brain selenium levels. However, restored selenium levels did appear to extend the duration of increased [DA]r in KOs, most likely through restored expression of one or more selenoproteins other than SELENOP1.

Next, we tested whether the methamphetamine-induced increase in [DA]r in SELENOP1 KO NAc slices could be prevented by pre-treatment with purified SELENOP1 protein. We applied SELENOP1 (100 pM) to brain slices via perfusion for 30 min immediately before methamphetamine application (Hollenbach et al., 2008). SELENOP1 protein by itself did not change DA release or uptake in either WT or SELENOP1 KO slices (**Figure 3A**). However, SELENOP1 suppressed the methamphetamine-induced increase in vesicular DA release in SELENOP1 KO slices, effectively rescuing the KO phenotype, without altering the response to methamphetamine in WT slices (**Figure 3B**). The change in [DA]r was significantly lower in SELENOP1-treated SELENOP1 KO slices than in non-treated SELENOP1 KO slices ($F_{(3,17)} = 2.284$, $p < 0.001$) (**Figure 3C**).

To determine whether the SELENOP1 was changing the methamphetamine responses by delivering selenium to NAc slices, we utilized a full-length (FL) all-Cys SELENOP1 mutant. All 10 Sec residues were changed to Cys residues in this mutant, eliminating the selenium content and preventing selenium delivery. Pre-treatment with the FL all-Cys SELENOP1 mutant to SELENOP1 KO slices resulted in a robust suppression of the methamphetamine-induced vesicular DA release, despite lacking selenium (**Figures 3D–F**). This demonstrates that SELENOP1 works through a selenium-independent mechanism to rescue the SELENOP1 KO phenotype.

The N-terminal domain contains several functional sites, including heparin and metal-binding regions and a redox motif. To determine if one of these properties could be responsible for the actions of SELENOP1 on slices, we utilized a mutant consisting of just the N-terminal domain fragment (NT) of the all-Cys SELENOP1 mutant. Pre-treating slices with the NT mutant resulted in an increase in [DA]r in response to methamphetamine comparable to untreated SELENOP1 KO slices (**Figures 3D–F**). The ineffectiveness of the NT mutant to rescue the SELENOP1 KO phenotype indicates that the SELENOP1 protein requires the C-terminal domain.

SELENOP1 binds to the apolipoprotein E receptor 2 (ApoER2) for selenium delivery. Other ApoER2 ligands such as reelin can initiate intracellular signaling (Bock and May, 2016). Previous studies have not addressed a potential role for SELENOP1 in ApoER2-mediated signaling. The ApoER2 binding site of SELENOP1 is in the C-terminal domain (Kurokawa et al., 2014). To explore the possibility that interaction of SELENOP1 with ApoER2 is a contributing factor, we used an all-Cys SELENOP1 mutant in which an essential region (residues 234–237) for ApoER2 binding is deleted, eliminating the ability of SELENOP1 to bind ApoER2 (Kurokawa et al., 2014). The mutated peptide without the ApoER2 domain (Δ234-237) did not prevent the methamphetamine-induced [DA]r increase (**Figures 3D–F**). One-way ANOVA revealed that a significant reduction in the [DA]r response to methamphetamine occurred only following pre-treatment with the FL all-Cys mutant ($F_{(3,11)} = 1.128$, $p < 0.05$). These data demonstrate that SELENOP1-ApoER2 interaction is required to attenuate the increased methamphetamine response in SELENOP1 KO slices.

D2R Activity Underlies Altered Methamphetamine Response in SELENOP1 KO NAc and Rescue by Purified SELENOP1 Protein

Amphetamines reportedly have an excitatory effect on DA neuron firing that is masked by D2R auto-inhibition (Shi et al., 2000). We therefore investigated if the substantial increase in [DA]r induced by methamphetamine in SELENOP1 KO mice was due to a change in presynaptic D2R. To determine whether increasing D2R activity would prevent the methamphetamine-induced [DA]r increase in SELENOP1 KO mice, we applied the selective D2R agonist quinpirole to SELENOP1 KO and WT slices for 15 min prior to and for the duration of methamphetamine exposure. Quinpirole activates presynaptic D2R to increase auto-inhibition of vesicular DA release to reduce evoked DA responses measured through FSCV. Exposure to 30 nM quinpirole for 15 min caused a similar decrease in evoked DA release in WT and SELENOP1 KO slices (**Figures 4A,B**). Methamphetamine increased the [DA]r in both WT and SELENOP1 KO slices following quinpirole application but did not restore the [DA]r to pre-quinpirole levels (**Figure 4B**). The maximum [DA]r reached in SELENOP1 KO slices as a percentage of original pre-quinpirole baseline was much smaller

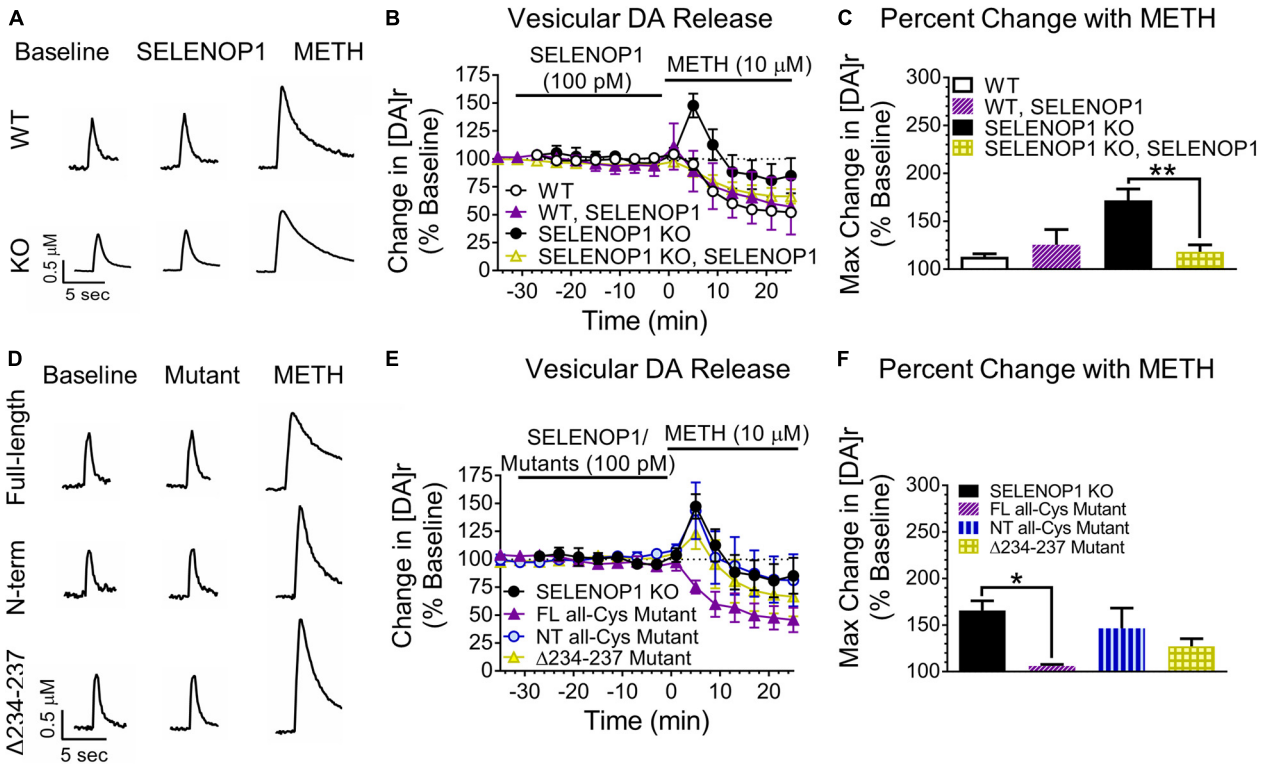

FIGURE 3 | SELENOP1 protein prevented methamphetamine-induced increases in vesicular DA release via ApoER2 receptor signaling. **(A)** sample traces from WT and SELENOP1 KO slices treated with purified SELENOP1 protein (100 pM) and subsequent methamphetamine exposure. **(B,C)** SELENOP1 protein reduced the [DA]r response to methamphetamine in SELENOP1 KO mice to a level comparable to WT mice (118.2 ± 7.3%; $n = 4$; One-way ANOVA: $F_{(3,17)} = 10.98$; Tukey's: $^{**}p = 0.0035$). SELENOP1 treatment had no effect on the WT response. **(D)** sample traces of SELENOP1 KO mice when treated with various SELENOP1 mutants (100 pM) before exposure to methamphetamine. **(E,F)** pre-treatment with a full-length all-Cys SELENOP1 mutant lacking selenium (FL all-Cys Mutant) was successful in preventing the methamphetamine-induced increase in [DA]r in SELENOP1 KO mice (106.0 ± 1.7%; $n = 3$; One-way ANOVA: $F_{(3,11)} = 4.586$; Tukey's $^{*}p = 0.0124$). Treatment with an all-Cys N-terminal region SELENOP1 peptide (NT all-Cys Mutant) lacking the C-terminus, however, did not prevent the increase in [DA]r in response to methamphetamine (146.5 ± 21.7%; $n = 3$). The Δ234-237 SELENOP1 mutant that is unable to bind ApoER2 (Δ234-237 Mutant) also did not reduce the [DA]r response (127.2 ± 8.2%; $n = 3$). All values reported are mean ± SEM.

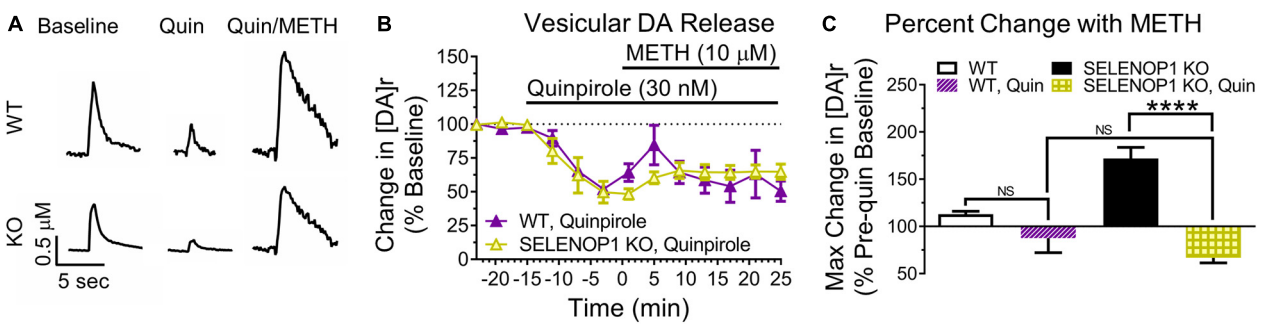

FIGURE 4 | The D2R agonist quinpirole prevented methamphetamine-enhanced vesicular DA release in SELENOP1 KO NAc. **(A)** representative DA signal traces of quinpirole-enhanced D2R auto-inhibition of evoked DA release from C57 WT mice and C57 SELENOP1 KO mice, aged 3–5 months. **(B)** quinpirole (Quin; 30 nM) reduced basal DA release in WT (–54.1 ± 2.8%; $n = 3$) and SELENOP1 KO (–58.4 ± 3.9%; $n = 3$) mice similarly ($p = 0.4$). Measurements followed 15 min of quinpirole exposure (last stimulation prior to 10 µM methamphetamine (METH) application). Quinpirole also suppressed the methamphetamine-induced increase in [DA]r in SELENOP1 KO mice. WT and SELENOP1 KO mice had comparable responses to methamphetamine following quinpirole. **(C)** mean (± SEM) changes in [DA]r in response to quinpirole and methamphetamine compared to pre-quinpirole baseline levels using a two-way ANOVA. Quinpirole reduced the methamphetamine responses in SELENOP1 KO slices (66.8 ± 5.3%; $n = 3$; $^{****}p < 0.0001$). Values shown here for WT and SELENOP1 KO groups without quinpirole are the same data previously shown in **Figure 3C**. All values reported are mean ± SEM.

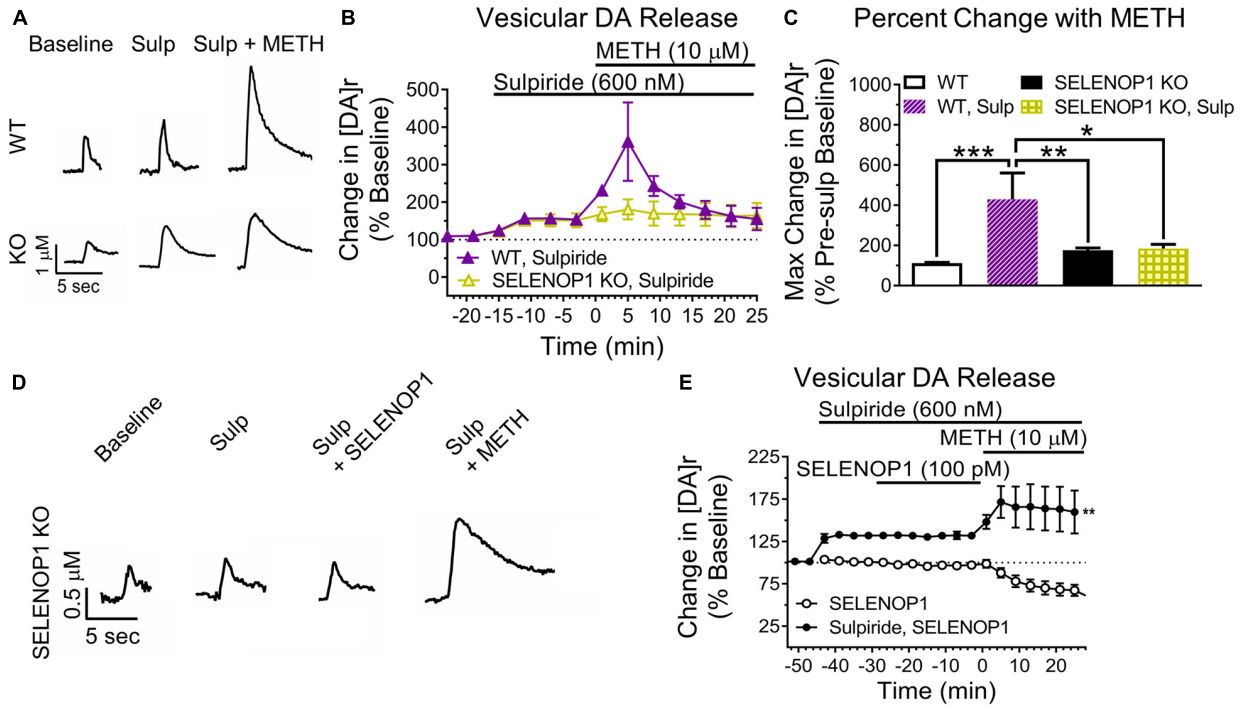

FIGURE 5 | D2R antagonism unmasked elevated vesicular DA release in WT while preventing rescue in KO mice. **(A)** shown are sample traces showing sulpiride (Sulp; 600 nM) reduction of D2R auto-inhibition and subsequent responses to 10 μM methamphetamine (METH). **(B)** sulpiride increased the baseline evoked DA release from baseline values in WT and SELENOP1 KO mice similarly (148.5 ± 4.6% and 139.8 ± 6.1%, respectively; $n = 4, 7$; $p = 0.4$). **(C)** sulpiride caused a dramatic increase in [DA]r in WT mice (429.3 ± 131.4%; $n = 3$; One-way ANOVA: $F_{(3,17)} = 10.72$; Tukey's $^{*}p = 0.0111$, $^{**}p = 0.0027$, $^{***}p = 0.002$), while resulting in no changes to subsequent methamphetamine responses in SELENOP1 KO mice (176.3 ± 10.4%; $n = 6$) compared to the SELENOP1 KO methamphetamine responses without sulpiride (171.9 ± 1.86%; $n = 6$). Data are expressed relative to baseline values before sulpiride application and compared to non-sulpiride methamphetamine experiment responses. Values shown here for WT and SELENOP1 KO groups without sulpiride are the same data previously shown in **Figure 3C**. **(D)** SELENOP1 KO mouse sample DA traces following application of sulpiride and SELENOP1 protein prior to methamphetamine. **(E)** sulpiride prevented SELENOP1 protein from suppressing the methamphetamine-induced increase in [DA]r in SELENOP1 KO mice (203.4 ± 23.9%; One-way ANOVA: $F_{(3,17)} = n = 4$; $^{**}p = 0.0069$). All values reported are mean ± SEM.

than the increase typically observed in SELENOP1 KO slices without quinpirole application (**Figure 4C**) ($F_{(3,16)} = 2.349$, $p < 0.0001$). WT and SELENOP1 KO slices treated with quinpirole exhibited comparable percent increases in [DA]r during methamphetamine application.

Next, we blocked D2R auto-inhibition with the D2R antagonist sulpiride. We predicted that sulpiride would unmask methamphetamine-enhanced vesicular DA release in WT slices. Sulpiride application (600 nM) increased evoked DA release similarly in both SELENOP1 and WT slices (**Figure 5A**). Methamphetamine exposure post-sulpiride application dramatically increased [DA]r in slices from WT mice, eliciting a more pronounced phenotype than what was observed in non-sulpiride exposed WT and SELENOP1 KO slices ($F_{(3,16)} = 2.445$, $p < 0.001$) (**Figures 5B,C**). The response in sulpiride-exposed WT slices was also larger than sulpiride-exposed SELENOP1 KO slices. Sulpiride with methamphetamine did not further increase [DA]r in SELENOP1 KO slices significantly above levels observed with methamphetamine alone.

Since sulpiride antagonism of D2R auto-inhibition unmasked a methamphetamine-induced increase in [DA]r in WT slices, we hypothesized that D2R antagonism would prevent the SELENOP1-induced rescue. To test this, we bath applied sulpiride (600 nM) prior to SELENOP1 protein, then followed by methamphetamine application. Sulpiride prevented the suppressive action of SELENOP1 protein, resulting in roughly a doubling of [DA]r over baseline once methamphetamine was added when compared to just SELENOP1-applied SELENOP1 KO slices ($t_{(6)} = 4.022$, $p < 0.001$) (**Figures 5D,E**). These results indicate the ability of SELENOP1 protein to directly reverse the SELENOP1 KO phenotype through an increase in D2R auto-inhibition (**Figure 6**).

DISCUSSION

We report decreased mesolimbic DA transmission, but increased vesicular DA exocytosis in response to methamphetamine, in SELENOP1 KO mice. In WT mice, a similar methamphetamine-induced increase in vesicular DA release was unmasked by blocking D2R receptor auto-inhibition. Furthermore, SELENOP1 modulated DA transmission through an ApoER2-dependent mechanism not involving selenium transport.

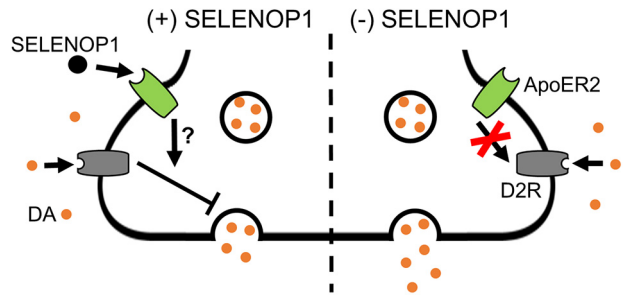

FIGURE 6 | Proposed mechanism for SELENOP1 modulation of DA release. Under conditions of DA overflow beyond the synaptic cleft, such as exposure to methamphetamine, excess DA activates D2R receptors to limit DA release. SELENOP1-ApoER2 interaction partially maintains D2R activity through a mechanism that remains to be characterized (left). This mechanism could involve a direct change in D2R function, surface expression, or cross-talk between ApoER2 and D2R intracellular signaling pathways. In the absence of SELENOP1-ApoER2 interaction (right), D2R activity is deficient, allowing for augmentation of vesicular DA release. Abbreviations: ApoER2, apolipoprotein E receptor 2; DA, dopamine; D2R, dopamine receptor 2; SELENOP1, selenoprotein P.

Electrically evoked DA signals were significantly reduced in slices from SELENOP1 KO mouse NAc compared to slices from WT mice, as shown by decreased peak extracellular DA concentration as well as a decrease in the early rising slope. These measurements agree with a decrease in total DA release, [DA]r, derived from the kinetic model. The difference between genotypes in the ratios of 2- and 10-pulse stimulation relative to single pulse (**Figures 1D,E**) may indicate a different release probability (Cragg, 2003; Condon et al., 2019). Following methamphetamine, the extracellular DA levels and DA release for KO slices were similar to pre-methamphetamine levels in WT slices (**Figure 2C**). This could indicate an overall reduction in synapse number or vesicles per terminal. A reduction in DA terminals would likely correlate with a reduction in DA terminal proteins such as DAT or DA vesicular proteins such as VMAT. However, the vesicular protein VMAT-2 expression was increased in the SELENOP1 KO ventral striatum, while expression of the presynaptic terminal protein DAT was unchanged, findings that are not consistent with a loss of dopaminergic terminals. Furthermore, our observation that the ratio of VMAT-2/DAT expression was increased in SELENOP1 KO ventral striatum suggests an increase rather than reduction in DA vesicles per terminal in the SELENOP1 KO NAc. Increased vesicles could be a consequence of decreased excitatory release that results in a build-up of releasable vesicles. Interestingly, the amplitude of DA release in response to multi-pulse stimulation increased over the response to single-pulse stimulation to a greater degree in SELENOP1 KO slices than in WT slices, suggesting a greater increase in vesicular release probability. Thus, SELENOP1 KOs may have a larger ratio of DA reserve vesicles to readily releasable vesicles in the NAc compared to WT mice. Basal DA uptake rates were reduced in SELENOP1 KO slices, which typically indicates lower DAT expression. Western blot analysis did not detect any change in DA expression in SELENOP1 KO striatum, however, which suggests that the functionality of DAT may be impaired in the SELENOP1 KO NAc under baseline conditions.

Although amphetamines are thought to primarily increase extracellular DA levels via reuptake blockade and non-vesicular release (Sulzer and Rayport, 1990; Sulzer et al., 1992; Seiden et al., 1993), some studies have suggested that amphetamines can increase vesicular release of DA (Covey et al., 2013, 2016; Daberkow et al., 2013). Covey et al. suggested that amphetamines up-regulate the readily releasable pool in ventral striatum to increase vesicular release (Covey et al., 2013). In this scenario, methamphetamine would mobilize DA to the readily releasable pool to increase the evoked DA signal and [DA]r. Thus, if a greater portion of DA is stored within the reserve pool in SELENOP1 KO mice and methamphetamine works by mobilizing this DA for release, then the mobilization of this pool of DA may contribute to the greater increase in DA release over baseline observed in SELENOP1 KO slices when methamphetamine is added. Our findings demonstrate a previously unreported function of SELENOP1 independent of selenium transport and other known properties. Supplementing SELENOP1 KO mice with selenium via drinking water showed the same peak response to methamphetamine as non-supplemented mice. Selenium supplementation did seem to mitigate the decay in vesicular DA release over time following the spike at the beginning of methamphetamine exposure, showing some effect, but it did not change the early kinetics. Selenium supplementation restores brain selenium levels and reverses selenium-related impairments (Hill et al., 2003; Schomburg et al., 2003; Nakayama et al., 2007). It is possible that the amount of selenium ingested via drinking water may have varied between each mouse. However, the variability of the data collected from selenium-supplemented mice was similar to that taken from non-supplemented mice with equal samples sizes (**Supplementary Figure 5**). Therefore, the altered methamphetamine response in the SELENOP1 KO mice does not appear to be due to reduced brain selenium levels. Neurodevelopmental changes in the DA system of SELENOP1 KO mice are possible as the SELENOP1 receptor ApoER2 facilitates DA neuronal migration during development (Sharaf et al., 2013, 2015). However, the observation that short-term application of SELENOP1 could restore the methamphetamine response to WT levels argues against major developmental impairments. Moreover, the full-length all-Cys SELENOP1 mutant lacking selenium was as effective as the non-mutated full-length SELENOP1 at restoring the methamphetamine response. The truncated N-terminal fragment was ineffective, however, ruling out several functions of the N-terminal domain. These include the antioxidant activity of the thioredoxin-like redox motif, the binding of heparin glycoproteins, and metal binding properties (Burk and Hill, 2015). Thus the C-terminal SELENOP1 domain, which includes the ApoER2 binding site (Kurokawa et al., 2014), is necessary for the observed changes in DA release. The Δ234-237 SELENOP1 mutant, with a specific deletion of the ApoER2-binding domain of SELENOP1, was also ineffective. This indicates that the interaction of SELENOP1 with ApoER2 is necessary to restore the suppressive response to methamphetamine. SELENOP1 co-localized with DAT in postmortem human brain, indicating

the presence of SELENOP1 at DA terminals (Bellinger et al., 2012). These results, taken together, provide strong evidence for SELENOP1-mediated signaling though ApoER2.

Previous studies showed that SELENOP1 binds to ApoER2 in order to mediate selenium transport across membranes (Burk et al., 2007, 2014; Olson et al., 2007). ApoER2 has a separate role in conjuction with the very-low-density-lipoprotein receptor (VLDLR) in mediating Reelin signaling (Reddy et al., 2011). Our results suggest that an additional role for SELENOP1-ApoER2 interaction is to induce a possible signal cascade to modulate DA release. ApoER2 interacts with different scaffolds and adaptor proteins, such as Dab1, which promotes ApoER2 surface expression, while ligands such as ApoE can promote ApoER2 internalization (Cuitino et al., 2005). Interestingly, the adaptor protein CIN85 binds to Dab1 to potentially mediate internalization of various membrane receptors, including D2R (Shimokawa et al., 2010; Fuchigami et al., 2013). This suggests a possible mechanism for which ApoER2 may be able to influence D2R surface expression. ApoER2 is also known to associate with the N-methyl-D-aspartate receptor (NMDAR). NMDAR activation on active pre-synaptic striatal DA terminals promotes DA release in a Ca^{2+}-dependent manner (Wang, 1991). Therefore, internalization of DA terminal-resident NMDARs, post-synaptic to regulatory glutamatergic inputs, via ApoER2 activation is another possible mechanism underlying the SELENOP1-dependent limitation of DA release.

Methamphetamine is thought to primarily increase extracellular DA levels via reuptake blockade and non-vesicular release (Sulzer and Rayport, 1990; Sulzer et al., 1992; Seiden et al., 1993). However, studies have suggested that amphetamines can increase vesicular release of DA (Covey et al., 2013, 2016; Daberkow et al., 2013). Covey et al. (2013) suggested that amphetamines up-regulate the readily releasable pool in ventral striatum to increase vesicular release. In this scenario, methamphetamine would mobilize DA to the readily releasable pool to increase the evoked DA signal and [DA]r.

We observed increased vesicular release not only in SELENOP1 KO mice, but also in WT animals in the presence of a D2R antagonist. Pre-application of the D2R antagonist sulpiride revealed a methamphetamine-induced increase in vesicular release in WT slices independent of DAT inhibition. Shi et al. (2000) reported that amphetamine causes an excitation in VTA DA neurons, which is masked by D2R activation via amphetamine-elevated DA concentrations. Thus, D2R autoreceptors may prevent the observation of increased vesicular release. It is worth noting that, in our experiments, pre-application of sulpiride did not potentiate the response to methamphetamine in SELENOP1 KO slices to as great of an extent as in WT slices. It is possible that this is because D2R autoreceptors are already unable to limit vesicular DA release in the SELENOP1 KO NAc in the presence of methamphetamine. Further interrogation of this relationship would benefit from including dose-response curves for these different pharmacological treatments and, thus, represents a limitation of the current study.

The prevention of a methamphetamine-induced increase in vesicular release in SELENOP1 KO phenotype by exogenous SELENOP1 likely involves D2R activity. The D2R agonist quinpirole prevented the large methamphetamine-induced increase of [DA]r in SELENOP1 KO slices. This finding implies reduced D2R activity in the SELENOP1 KO NAc, which is accentuated in the context of methamphetamine exposure. Sulpiride prevented SELENOP1 protein from increasing stimulated DA release in SELENOP1 KO slices, suggesting activation of a signaling pathway that restores D2R activity and limits increases in vesicular DA release. This pathway appears to involve SELENOP1-ApoER2 interaction, as a mutation to the ApoER2-binding domain of SELENOP1 prevented the rescue of the KO phenotype. Taken together, these results suggest that SELENOP1-ApoER2 binding normally promotes D2R function, likely auto-inhibitory, which masks the methamphetamine enhancement of vesicular DA release. In the absence of SELENOP1, D2R activity may be decreased, allowing for the large increases in [DA]r we observed. This proposed mechanism is summarized in **Figure 6**. Further investigation is needed to determine the pathways through which ApoER2 regulates D2R. Among the possibilities are (1) changes in D2R surface expression, (2) changes in D2R functionality, and (3) cross-talk between ApoER2 and D2R intracellular signaling pathways. Interestingly, mice with heterozygous genetic deletion of the ApoER2 ligand Reelin exhibit region-specific alterations in D2R expression, with both increases and decreases reported occurring in the striatum (Varela et al., 2015).

The results described herein directly implicate SELENOP1 as an important regulator of DA transmission, a role not previously reported. In contrast to several studies that have reported elevated DA turnover in rats in response to dietary selenium restriction (Castano et al., 1997; Rasekh et al., 1997; Romero-Ramos et al., 2000), we demonstrate decreased basal DA release in SELENOP1 KO mouse striatal slices. The previous reports are not necessarily in conflict with our findings, however, as these studies reported DA and DA metabolites measured over longer periods of time (hours and days) compared our study (minutes in duration with sub-second temporal resolution). Elucidating this relationship sheds further light on the protective actions of selenium against methamphetamine-induced neurotoxicity (Imam et al., 1999; Kim et al., 1999; Barayuga et al., 2013) by demonstrating the ability SELENOP1 to limit extracellular DA transmission. This can potentially limit damage to dopaminergic terminals caused by excessive DA auto-oxidation that result from excessive dopaminergic activity, such as that caused by methamphetamine (Cadet and Brannock, 1998). The current study also improves our understanding of the methamphetamine mechanism of action as it provides corroborating evidence that methamphetamine increases vesicular DA release, a phenomenon reported for amphetamine in several previous studies (Covey et al., 2013, 2016; Daberkow et al., 2013). Amphetamine-induced elevations in extracellular DA in rodent NAc slices are dependent on DAT (Siciliano et al., 2014). However, the measured increases in [DA]r observed in this study are likely independent of DAT inhibition, as methamphetamine-influenced reuptake kinetics in SELENOP1 KO slices were comparable to WT slices. These data may be relevant to addiction since DA release events are critical in reward-based learning and drug reinforcement

(Stuber et al., 2005; Steinberg et al., 2014), and the NAc shell is thought to play a more significant role in addiction compared to the NAc core (Ikemoto, 2007).

We previously reported the association of SELENOP1 with lesions of both Alzheimer's disease (Bellinger et al., 2008) and Parkinson's disease (Bellinger et al., 2012), suggesting a role in neurodegeneration. Given that dopaminergic terminals are particularly vulnerable to damage such as that from DA auto-oxidation, the demonstrated ability of SELENOP1 to limit DA release raises the possibility of a neuroprotective role in neurodegenerative diseases and aging (Kumar et al., 2012). Importantly, ApoER2 is also a receptor for ApoE, for which the e4 polymorphism is the most prominent genetic risk factor for Alzheimer's disease (Zhao et al., 2018). One possibility is that ApoE limits the protective influence of SELENOP1 by competing for ApoER2 binding or reducing ApoER2 surface expression (Chen et al., 2010). In addition to Alzheimer's disease, ApoE has been implicated in other diseases such as parkinsonism (Jellinger, 2018) and HIV-related dementia (Olivier et al., 2018), further highlighting SELENOP1-ApoER2 interaction as an area of interest in neurodegeneration research.

This study demonstrates dopaminergic regulation by SELENOP1. We show that genetic deletion of SELENOP1 results in increased DA vesicular release in response to methamphetamine, and that addition of exogenous SELENOP1 prevents this increase. The direct actions of SELENOP1 involve (1) binding to ApoER2 and (2) D2R activity. Furthermore, we demonstrate that D2R receptor auto-inhibition masks an increase in vesicular DA release in WT mice. Our findings show that SELENOP1 can act to modulate neurotransmission through a mechanism other than selenium delivery, further expanding its role in the brain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Hawai'i at Mânoa Institutional Animal Care and Use Committee (UH Manoa IACUC).

AUTHOR CONTRIBUTIONS

FB, DT, JY, SS, SK, and MA designed the research. DT, CM, and AH performed the research. DT, JY, CM, and FB analyzed the data. DT and FB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.631825/full#supplementary-material

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Stress and the Brain: An Emerging Role for Selenium

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The stress response is an important tool in an organism's ability to properly respond to adverse environmental conditions in order to survive. Intense acute or chronic elevation of glucocorticoids, a class of stress hormone, can have deleterious neurological effects, however, including memory impairments and emotional disturbances. In recent years, the protective role of the antioxidant micronutrient selenium against the negative impact of externally applied stress has begun to come to light. In this review, we will discuss the effects of stress on the brain, with a focus on glucocorticoid action in the hippocampus and cerebral cortex, and emerging evidence of an ability of selenium to normalize neurological function in the context of various stress and glucocorticoid exposure paradigms in rodent models.

Keywords: selenium, stress, selenocompounds, glucocorticoids, selenoproteins

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INTRODUCTION

The impact of stress on human health has been extensively investigated and the role of stress in disease pathology has become apparent over recent decades (Chrousos, 2009). The brain plays a key role in the response to stress, which includes higher order processing of stress-related information and an immediate physiological response executed by the hypothalamus, the proverbial "fight or flight response." In addition to direct autonomic input to specific tissues, the stress response involves sending hormonal signals throughout the body via the hypothalamic-pituitary-adrenal (HPA) axis (depicted in **Figure 1**). Signaling along this pathway begins with the release of corticotropin-releasing hormone (CRH) by neurosecretory cells in the paraventricular nucleus of the hypothalamus. Upon stimulation by CRH, the anterior pituitary releases adrenocorticotropin-releasing hormone (ACTH), which then induces adrenal gland secretion of glucocorticoids into the bloodstream. Glucocorticoids comprise the main downstream component of the neuroendocrine response to stress and primarily serve to stimulate gluconeogenesis in the liver and lipolysis for energy production. They also suppress the inflammatory actions of the immune system and, thus, synthetic glucocorticoids are commonly prescribed in humans as anti-inflammatory medications. The autonomic component of the stress response, which includes vasoconstriction, inducing perspiration, and suppressing digestive activity, works in conjunction with glucocorticoids to provide an acute adaptation to stressful stimuli. Glucocorticoid receptors (GCR) are expressed in most tissues in mammals, however, and the physiological processes affected are wide-ranging. For example, both the hypothalamus and anterior pituitary express GCRs to provide negative feedback loops within the HPA axis by suppressing CRH and ACTH production (Godoy et al., 2018).

The brain is particularly sensitive to glucocorticoid levels and both acute and chronic stress (e.g., brief incidence of high stress or long-term exposure to low or moderate stress) can have deleterious effects on neurological function, including depressive symptoms and memory problems

(McEwen, 2008; Lupien et al., 2018). In recent years, pre-clinical studies have demonstrated that the antioxidant micronutrient selenium has the capacity to alleviate the neurological repercussions of stress and exogenous glucocorticoid exposure. This review will provide an overview of the negative impact of stress on the brain, with a focus on glucocorticoid activity, and discuss the emerging evidence of the protective nature of selenium.

STRESS AND THE BRAIN

The reaction to stress, whether psychological or physical, can be defined as an attempt to regain homeostasis following a disruptive environmental stimulus (Chrousos, 2009). The short-term neuroendocrine response to stress provides adaptive benefits, but prolonged and repeated activation causes physiological "wear and tear" throughout the body, including the brain (McEwen, 2007). Excessive exposure of the brain to cortisol, which is the main active glucocorticoid in humans and can easily pass the blood–brain barrier, leads to deficits in learning and memory, attention, and emotional disturbances (Lupien et al., 2009). These neurological impairments are linked to dysfunction of the prefrontal cortex, the hippocampus, and the amygdala, brain structures that are integral to the processing of stress-related information and are particularly responsive to glucocorticoids (Lupien and Lepage, 2001). Following the discovery by McEwen et al. (1968) that corticosterone, the main active glucocorticoid in rodents, can act on the rat brain, it was noted that the hippocampus has the highest density of GCRs. Subsequently, the effects of glucocorticoids on the hippocampus and the relation to stress-induced cognitive dysfunction have been extensively characterized in animal and human studies throughout the years (Lupien and Lepage, 2001; McEwen et al., 2016; Lupien et al., 2018).

There are various ways that stress and glucocorticoid exposure can damage the brain. Early research in the field indicated that GCR over-activation causes neuronal damage by disrupting energy production, promoting energy over-consumption, and limiting glucose uptake into the cell (Sapolsky, 1986). Additionally, glucocorticoids can increase the risk of excitotoxicity by promoting the extracellular accumulation of glutamate in the hippocampus and prefrontal cortex (Stein-Behrens et al., 1994; Treccani et al., 2014). Oxidative stress is a prominent mediator of neuronal damage and dysfunction caused by psychological stress paradigms and exogenous glucocorticoid administration in rodents (Spiers et al., 2014). Glucocorticoids appear to make neurons more susceptible to oxidative insult by raising baseline levels of reactive oxygen species (ROS; McIntosh and Sapolsky, 1996; Behl et al., 1997).

SELENIUM AND SELENOPROTEINS IN BRAIN HEALTH

The antioxidant trace element selenium is vital for overall human health and is especially important for brain function. Within the brain, selenium protects against oxidative stress, endoplasmic reticulum stress, and inflammation. There is also evidence that this micronutrient supports neurotransmission by maintaining redox balance (Solovyev, 2015). Selenium must be acquired through the diet and is most abundant in meats and legumes, as well as fruits and vegetables in trace amounts (Navarro-Alarcon and Cabrera-Vique, 2008). In the mammalian body, selenium is used to synthesize the amino acid selenocysteine (Sec), to be incorporated into selenoproteins, of which there are 25 types present in humans. Among the most well-characterized selenoproteins is the glutathione peroxidase (GPx) sub-family, responsible for reducing peroxide species, the thioredoxin reductases (TrxnR), and the iodothyronine deiodinases (Dio), which support thyroid hormone metabolism. In general, adequate selenoprotein expression largely depends on an organism's intake of selenium, which is preferentially retained within the brain (Burk and Hill, 2009). Selenoprotein P (SelenoP), which is unique in that it has 10 Sec residues rather one, acts as a selenium carrier (Labunskyy et al., 2014). Following its secretion from the liver, SelenoP travels through the blood stream to be delivered to critical organs, such as the brain, where it interacts with apolipoprotein e receptor 2 (ApoER2) to deliver selenium (Burk et al., 2014). The brain is particularly dependent on selenium due to high rates of oxygen consumption and heightened susceptibility to oxidative stress (Steinbrenner and Sies, 2013). Insufficient selenium supply and lack of selenoprotein function have been linked to multiple brain disorders, including neurodegenerative diseases, which have been thoroughly discussed in previous reviews (Pillai et al., 2014; Solovyev, 2015; Varikasuvu et al., 2019; Zhang et al., 2019). Conversely, selenium has been suggested as a potential therapeutic agent in the treatment of Alzheimer's disease (Solovyev et al., 2018), multiple sclerosis (de Toledo et al., 2020), and stroke (Alim et al., 2019).

Throughout the body, glucocorticoids have shown a capacity to alter antioxidant enzyme activity and expression (Dougall and Nick, 1991; Asayama et al., 1992; Kratschmar et al., 2012; An et al., 2016). In the brain, glucocorticoids can down-regulate several types of antioxidant enzymes, including GPx (McIntosh et al., 1998; Sahin and Gumuslu, 2004; You et al., 2009; Sato et al., 2010). Over the past several years, selenium has been shown to mitigate the negative impact of stress and glucocorticoid action in the brain.

SELENIUM AND GLUCOCORTICOID ACTION IN THE BRAIN

A literature review was conducted with Web of Science and PubMed using the words "selenium" or "selenoprotein" combined with either "glucocorticoid" or "corticosterone," as well as either with or without "brain", yielding the following information. Early studies associating selenium and glucocorticoids focused on the physiological response to acute selenium challenge. Researchers discovered that injection of sodium selenite provokes a stress response, raising plasma corticosterone and glucose levels in rats within 30 min

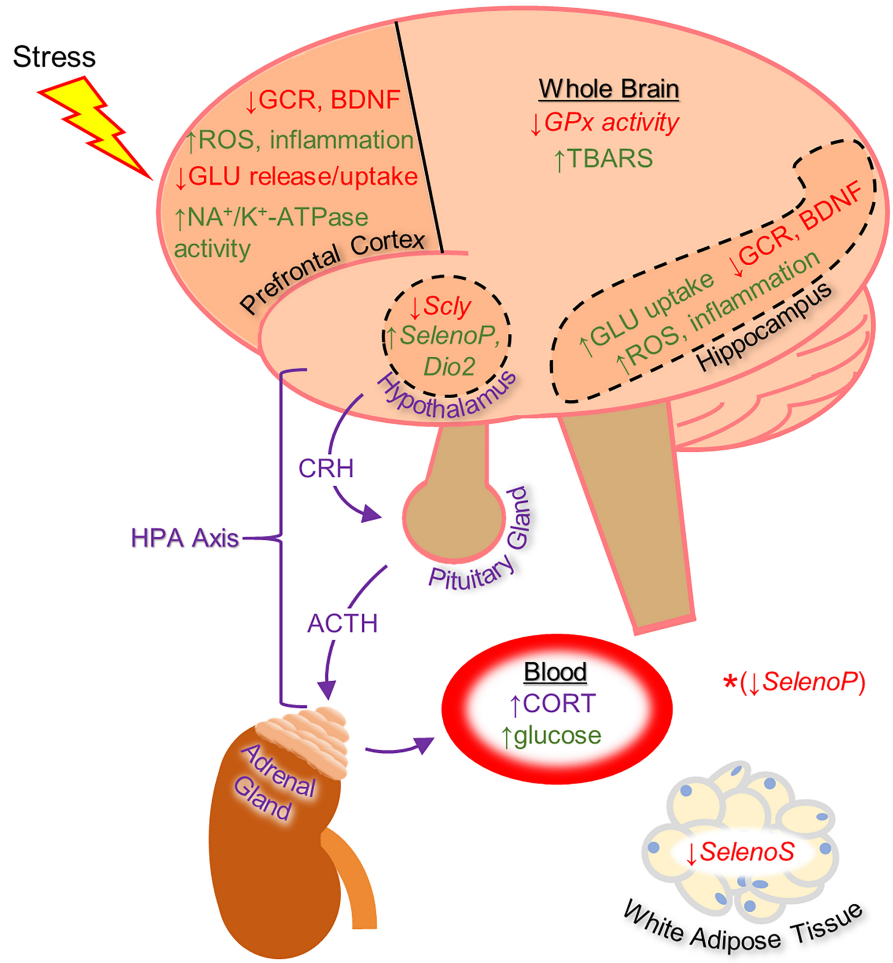


FIGURE 1 | Diagram of the effects of heightened stress on the brain and other tissues as well as on the function of the hypothalamic-pituitary-adrenal (HPA) axis (in purple), based mainly on rodent studies. The effects shown include regulation of selenoprotein expression by glucocorticoid administration (shown with italicized text). *The synthetic glucocorticoid dexamethasone was shown to down-regulate Selenoprotein P gene expression in HEK-293 human embryonic kidney cells. With the exception of changes to selenoprotein expression, the effects listed on this diagram were shown to be reversible by selenium-based therapy. Up-regulation is indicated by green text and down-regulation by red text. ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; CORT, corticosterone; CRH, corticotropin-releasing hormone; Dio2, Iodothyronine deiodinase 2; GCR, glucocorticoid receptor; GLU, glutamate; GPx, glutathione peroxidase; ROS, reactive oxygen species; Scly, selenocysteine lyase; SelenoP, Selenoprotein P; SelenoS, Selenoprotein S; TBARS, thiobarbituric acid reactive substances.

(Rasekh et al., 1991; Potmis et al., 1993). These studies established that acute elevations in selenium supply can activate the HPA axis. Conversely, dietary selenium deficiency blunts the ability of the adrenal gland to secrete corticosterone in response to ACTH administration (Chanoine et al., 2004). In addition to facilitating HPA axis activity, it was subsequently shown by Yilmaz et al. (2006) that selenium supplementation can reduce oxidative damage caused by the synthetic glucocorticoid prednisolone in the rat liver by maintaining reduced glutathione. More recent work by Beytut et al. (2018) found that pre-supplementation with sodium selenite prevented the rise in thiobarbituric acid reactive substances (TBARS) levels in rat brain caused by prednisolone injection. The authors hypothesized that glucocorticoids cause damage to neurons by inducing lipid peroxidation and that this occurs, at least in part, due to the ability of glucocorticoids to reduce antioxidant enzyme defense.

Work by Xu et al. (2020) suggests that dietary selenium may protect against stress-induced depressive symptoms. In this study, rats were subjected to social stress using a Chronic Unpredictable Mild Stress (CUMS) paradigm. While some developed depressive-like behavior and were classified as CUMS-sensitive, others did not and were, therefore, labeled CUMS-resilient. Analysis of trace element levels revealed that plasma selenium levels were lower in the CUMS-sensitive group, correlating low selenium levels with heightened susceptibility to stress-induced depressive-like symptoms. Additionally, an epidemiological study correlated low selenium intake with an increased susceptibility for developing major depressive disorder in humans (Pasco et al., 2012). It is important to note that these studies don't show cause and effect, however. Still, the effects of selenium intake on the response to stress or glucocorticoid administration remains largely under-investigated.

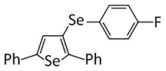
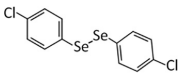
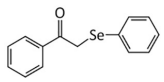
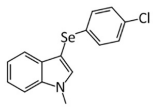
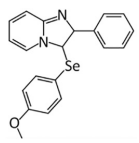
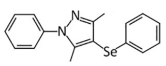
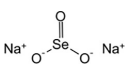
Over the past several years, the protective role of selenium against the neurobehavioral consequences of glucocorticoids has started to come to light. In 2014, a report by Gai et al. (2014) described the ability of 3-(4-fluorophenylselenyl)-2,5-diphenylselenophene (F-DPS) to alleviate the anxiogenic- and depressive-like symptoms induced by chronic corticosterone administration in male Swiss mice. The organoselenium compound F-DPS is a selenophene, a class of selenium-containing aromatic compounds with antioxidant properties (Wilhelm et al., 2009; Tavadyan et al., 2017; Manikova et al., 2018), and was chosen for its antidepressant-like properties (Gay et al., 2010). One week of F-DPS treatment reversed the depressant- and anxiogenic-like behavior induced by 4 weeks of corticosterone administration. Glutamate uptake in the prefrontal cortex was reduced by corticosterone, which the authors noted was consistent with previous studies (Gourley et al., 2012) and likely contributed to the depressive-like phenotype. Administration of F-DPS during the final week of corticosterone administration restored glutamate uptake in the prefrontal cortex without causing any changes in vehicle-treated mice. These results parallel findings from clinical studies demonstrating the anti-depressive effects of the glutamatergic NMDA receptor antagonist ketamine (Yang et al., 2019). Additionally, F-DPS treatment was shown to reduce hippocampal serotonin uptake and monoamine oxidase A activity. Thus, promotion of serotonergic activity may have also contributed to the anti-depressive action of F-DPS (Gay et al., 2010; Gai et al., 2012). The authors concluded that these effects in the brain may have been mediated by an ability of F-DPS to normalize HPA axis function, as it was shown to reverse the rise serum corticosterone levels (Gai et al., 2014).

Following the work by Gai et al. (2012), another study explored the relationship between selenium and glucocorticoids in relation to memory. Zborowski et al. (2016) evaluated the potential of 4,4′-dichloro-diphenyl diselenide $(p\text{-ClPhSe})_2$, an organoselenium compound with antidepressant and memory enhancing properties (Gai et al., 2012) to alleviate the memory impairments caused by exogenous corticosterone. The researchers found that treatment with $(p\text{-ClPhSe})_2$ improved the performance of corticosterone-exposed mice in several memory tasks, while normalizing glutamate uptake in hippocampal slices. Intriguingly, there were no signs of toxicity caused by $(p\text{-ClPhSe})_2$, a common concern with selenium-based therapies, supporting the therapeutic potential of $(p\text{-ClPhSe})_2$. Later work revealed that $(p\text{-ClPhSe})_2$ is effective in reversing the depressive-like phenotype induced by chronic dexamethasone injections in mice (Heck et al., 2019). In this study, Heck at al. chose the synthetic glucocorticoid dexamethasone because it is widely prescribed as an anti-inflammatory in humans. In addition to preventing dexamethasone-induced depressive-like behavior and reducing ROS levels in the prefrontal cortex, $(p\text{-ClPhSe})_2$ normalized glutamatergic uptake in the prefrontal cortex, further implicating glutamatergic neurotransmission as a significant factor in the protective actions of selenium.

Several studies have also investigated the protective effects of selenium using an acute restraint stress (ARS) paradigm (Buynitsky and Mostofsky, 2009). This paradigm typically involves immobilizing subjects in a plexiglass restraint device with the goal of causing stress while minimizing pain. Previous research indicates that ARS works in part by targeting the antioxidant and inflammatory capacity of the brain (Sosnovskii et al., 1993; Spiers et al., 2016; Sayd et al., 2020). In a 2018 report, Sousa et al. described the ability of the selenocompound α-(phenylselanyl) acetophenone (PSAP) to counteract the effects of ARS (Sousa et al., 2018). Previous studies demonstrated that PSAP has GPx-like antioxidant activity (Cotgreave et al., 1992) and antidepressant-like capabilities in mice (Gerzson et al., 2012). Sousa and colleagues immobilized mice in a restraint device for 4 h, followed by a battery of behavioral tests 40 min later. A single dose of PSAP administered just after ARS and prior to behavioral testing reversed all of the behavioral changes induced by ARS, which included depressive-like and anxiogenic-like behavior, as well as an elevated sensitivity to pain. Administration of PSAP also decreased lipid peroxides and ROS in the hippocampus and cerebral cortex, which became elevated in response to ARS. Finally, PSAP prevented the rise in serum corticosterone caused by ARS, mimicking the results from previous studies indicating that selenium has a "normalizing" effect on HPA axis activity.

Several other selenocompounds have shown promising effects in stressed mice. Casaril et al. (2019) showed that 3-((4-chlorophenyl)selanyl)-1-methyl-1H-indole (CMI) can prevent ARS-induced depressive-like behavior in mice without affecting non-stressed subjects. Originally developed to combat atherosclerosis-associated inflammation by protecting extracellular matrix proteins from oxidative stress, CMI induces antinociceptive effects in mice by modulating serotonergic activity (Casaril et al., 2017b) and can reverse the depressive-like phenotype caused by lipopolysaccharide injection (Casaril et al., 2017a). Casaril identified multiple oxidative and inflammatory pathways that were activated by ARS and which CMI attenuated. The authors also revealed that CMI reversed the down-regulation of GCR expression in the prefrontal cortex and hippocampus caused by ARS that may have impaired the negative feedback loop of glucocorticoid secretion. Subsequent research by Pesarico et al. (2020) revealed that CMI also prevents the depressive-like phenotype caused by repeated forced swimming. The authors hypothesized that CMI acted by reducing lipid peroxidation in the prefrontal cortex and hippocampus. Domingues et al. (2019) obtained similar results while treating ARS-exposed mice with 3-[(4-methoxyphenyl) selanyl]-2-phenylimidazo[1,2-a] pyridine (MPI), a selenocompound with antioxidant and anti-inflammatory properties in the brain (Domingues et al., 2018). Administration of MPI attenuated the depressive- and anxiety-like phenotypes caused by ARS while preventing the induction of pro-inflammatory markers. Using a molecular docking simulation, the authors revealed that MPI may be capable of binding the GCR directly. Finally, Birmann et al. (2021) showed that yet another selenocompound, 3,5-dimethyl-1-phenyl-4-(phenylselanyl)-1*H*-pyrazole (SePy), protects against the anxiogenic-like and hyperalgesic effects of ARS. The authors reported that SePy, which has anti-depressive-like properties (Birmann et al., 2020), prevented the ARS-induced elevation of TBARS levels in the prefrontal cortex and hippocampus,

TABLE 1 | Summary of the effects of selenium-containing compounds used in rodent models of stress.

Selenocompound/Species	Therapeutic Effects Against Stress in Rodent Studies		
 3-(4-Fluorophenylselenyl)-2,5-diphenylselenophene (F-DPS) (Gay et al., 2010)	(Gai et al., 2014) - Reversed depressant- and anxiety-like behaviors caused by CORT administration - Normalized serum ACTH and CORT levels - Lowered monoamine oxidase-A activity in the PFC - Augmented synaptosomal serotonin and restored GLU uptake in PFC		
 4,4′-dichloro-diphenyl diselenide (p-ClPhSe)$_2$ (Gai et al., 2012)	(Zborowski et al., 2016) - Restored spatial and non-spatial memory dysfunction caused by CORT administration - Reversed GLU uptake augmentation in HPC slices	(Heck et al., 2019) - Prevented depressive-like behavior induced by dexamethasone administration - Reduced ROS; Restored CAT, SOD activity. - Restored GLU uptake and release; reversed elevation of NA^+/K^+-ATPase activity in PFC.	
 α-(phenylselanyl) acetophenone (PSAP) (Gerzson et al., 2012)	- (Sousa et al., 2018) Prevented depressive- and anxiety-like behavior caused by ARS - Prevented the associated elevation in pain sensitivity and allodynia (perceiving normally non-painful stimuli as painful) - Normalized serum CORT levels - Reduced ROS, lipid peroxidation, nitrite, and nitrate levels in the CC, HPC		
 3-((4-chlorophenyl)selanyl)-1-methyl-1H-indole (CMI) (Vieira et al., 2015; Casaril et al., 2017b)	- (Casaril et al., 2019) - Prevented depressive-like behavior caused by ARS - Normalized serum CORT levels - Reduced ROS, lipid peroxidation, and nitric oxides in the PFC, HPC - Restored CAT activity in the HPC - Prevented down-regulation of GCR and BDNF, and up-regulation of inflammation in the PFC and HPC		- (Pesarico et al., 2020) - Prevented depressive-like behavior caused by the repeated forced swimming test - Normalized serum CORT levels - Prevented TBARS elevation in HPC - Restored SOD activity in HPC - Reversed the up-regulation of CAT activity in the PFC and down-regulation in the HPC
 3-[(4-methoxyphenyl) selanyl]-2-phenylimidazo[1,2-a] pyridine (MPI) (Domingues et al., 2018)	- (Domingues et al., 2019) - Prevented anxiogenic-like behavior caused by ARS - Normalized plasma CORT levels - Prevented the rise in plasma glucose levels - Prevented elevation of TBARS, ROS, and nitrate/nitrites in the PFC and HPC - Prevented elevation of inflammatory markers in the PFC and HPC - Prevented the down-regulation of BDNF in the PFC and HPC - May be capable of binding GCR directly		
 3,5-dimethyl-1-phenyl-4-(phenylselanyl)-1H-pyrazole (SePy) (Birmann et al., 2020)	- (Birmann et al., 2021) - Attenuated anxiety-like behavior, allodynia, and hyperalgesia caused by ARS - Normalized plasma CORT levels - Reversed the elevation of ROS and TBARS in the PFC and HPC - Restored SOD activity in the PFC and HPC - May be capable of binding GCR directly		
 Sodium Selenite (Na_2SeO_3)	- (Beytut et al., 2018) - Reduced total brain TBARS induced by prednisolone administration - Restored brain GPx activity and levels of reduced GSH - Did not, however, prevent the reduction in CAT activity		

*(**Left column**) Selenium-containing compounds used in the reviewed studies and references for preceding studies with those compounds. (**Right column**) Ameliorative effects of treatment with the selenocompounds against the neurological and physiological impact of various stress paradigms. ACTH, adrenocorticotropic hormone; ARS, acute restraint stress; BDNF, brain-derived neurotrophic factor; CAT, catalase; CC, cerebral cortex; CORT, corticosterone; GCR, glucocorticoid receptor; GLU, glutamate; GPx, glutathione peroxidase; GSH, glutathione; HPC, hippocampus; PFC, prefrontal cortex; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.*

while reducing plasma corticosterone levels. Additionally, SePy was predicted to bind the active site of GCRs, similar to MPI, using a computational model. The molecular effects of stress on the brain examined by these studies, as well as the impact on selenoprotein expression as discussed below, are summarized in **Figure 1**.

GLUCOCORTICOID REGULATION OF SELENOPROTEINS

Glucocorticoids can regulate selenoprotein expression as reported by a handful of studies. For example, Rock and Moos identified a retinoid responsive element that can be regulated by dexamethasone to decrease *SelenoP* expression in HEK-293 cells (Rock and Moos, 2009). In another report by Kim and Kim (2013), dexamethasone was found to induce proteasomal degradation of Selenoprotein S (SelenoS) in 3T3-L1 murine preadipocytes, which the authors identified as necessary for adipogenesis. These studies highlight the diverse mechanisms through which glucocorticoids may differentially regulate selenoprotein expression in a tissue-specific manner.

Our knowledge of the ability of glucocorticoids to regulate the selenoproteins was recently expanded to the brain by Wray et al. (2019). In this study, chronic corticosterone administration increased gene expression of *SelenoP* and *Dio2*, while decreasing expression of the selenium recycling enzyme selenocysteine lyase (*Scly*), in the arcuate nucleus (Arc) of the hypothalamus, a brain region with high GCR expression. The authors focused on the metabolic effects of glucocorticoids, which include over-eating and excess weight gain (Vegiopoulos and Herzig, 2007; Perez et al., 2014). Interestingly, elevated serum SelenoP has been associated with diabetes and obesity (Misu et al., 2010) and Dio2 increases hypothalamic thyroid hormone availability (Bechtold and Loudon, 2007) to promote food intake (Coppola et al., 2007; Ishii et al., 2008; Varela et al., 2012). The finding that corticosterone down-regulated *Scly* draws an interesting parallel to whole-body Scly knockout mice, which exhibit an over-weight phenotype and heightened susceptibility to developing metabolic syndrome (Seale et al., 2012, 2015). Thus, long-term glucocorticoid action may promote positive energy balance, in part, by altering the expression of Scly and the selenoproteome in the Arc and other parts of the hypothalamus. In light of these findings, investigation of the interactions between glucocorticoids and selenium within the hypothalamus, and the relation to stress-related metabolic disruptions as well as downstream HPA axis function, remains a worthy course of investigation.

DISCUSSION

The majority of studies characterizing the protective role of selenium against stress and exogenous glucocorticoid administration have utilized various selenocompounds that were previously shown to have antioxidant activity. While the relative contributions of the selenium residues within each of these compounds to the overall therapeutic effect observed is not immediately clear, the protective results reported by the studies reviewed herein are striking (reviewed in **Table 1**). Developing synthetic compounds that incorporate selenium may, in fact, be a useful alternative to dietary selenium supplementation by providing the potential for tissue-specific targeting and limiting cytotoxicity. Still, dietary selenium remains an attractive potential treatment to counteract the oxidative effects of glucocorticoid action due its ease of delivery, and broad availability as an over-the-counter supplement. A comprehensive investigation of the role of selenium in the brain in response to stress, as well as the influence of glucocorticoid activity on the broader selenoproteome, however, is merited as this remains a major research gap. Additionally, investigating the apparent capability of seleno-therapy to normalize HPA axis function is instructive in order to understand the overall physiological implications. In conclusion, the interactions between glucocorticoids and selenium represent an emerging field with exciting potential for therapeutic development.

AUTHOR CONTRIBUTIONS

DT and NA wrote the manuscript. All authors revised the manuscript.

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BRIEF RESEARCH REPORT
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Juvenile Selenium Deficiency Impairs Cognition, Sensorimotor Gating, and Energy Homeostasis in Mice

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Selenium (Se) is an essential micronutrient of critical importance to mammalian life. Its biological effects are primarily mediated via co-translational incorporation into selenoproteins, as the unique amino acid, selenocysteine. These proteins play fundamental roles in redox signaling and includes the glutathione peroxidases and thioredoxin reductases. Environmental distribution of Se varies considerably worldwide, with concomitant effects on Se status in humans and animals. Dietary Se intake within a narrow range optimizes the activity of Se-dependent antioxidant enzymes, whereas both Se-deficiency and Se-excess can adversely impact health. Se-deficiency affects a significant proportion of the world's population, with hypothyroidism, cardiomyopathy, reduced immunity, and impaired cognition being common symptoms. Although relatively less prevalent, Se-excess can also have detrimental consequences and has been implicated in promoting both metabolic and neurodegenerative disease in humans. Herein, we sought to comprehensively assess the developmental effects of both Se-deficiency and Se-excess on a battery of neurobehavioral and metabolic tests in mice. Se-deficiency elicited deficits in cognition, altered sensorimotor gating, and increased adiposity, while Se-excess was surprisingly beneficial.

Keywords: selenium, sensorimotor gating, cognition, energy metabolism, neurodevelopment

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INTRODUCTION

Selenium (Se) is an essential trace element in mammals, of which both deficiency and excess can have detrimental effects on health (1). Se supplementation within a narrow range optimizes activity of Se-dependent antioxidant enzymes that incorporate Se co-translationally in the form of selenocysteine. Se also counteracts the toxicity of certain heavy metals, such as arsenic, lead, and mercury (2, 3). Deficient Se intake impairs thyroid hormone metabolism and reduces activity of the antioxidant enzymes, glutathione peroxidase and thioredoxin reductase (4). In contrast, Se can be detrimental at high doses, with documented neurotoxic effects (5).

Se-deficiency is estimated to occur in roughly 10% of the world's population and is observed predominantly in regions with low soil Se-content, such as Scandinavia, New Zealand, and Northeast China (6). Furthermore, future climate change is predicted to decrease soil Se content in agricultural regions and augment the prevalence of Se-deficiency worldwide (7). Common symptoms associated with Se-deficiency include hypothyroidism, cardiomyopathy, compromised

immunity, fatigue, and cognitive deficits (8–10). Also, altered serum Se levels have been documented in both autism and schizophrenia (11, 12), and it is hypothesized that redox imbalance during neurodevelopment increases risk for these neuropsychiatric conditions (13, 14).

On the opposite end of the spectrum, Se-excess can lead to toxicity and increased oxidative stress. In rodents, acute Se overexposure elicits motor deficits, catalepsy-like behavior and increased levels of dopamine, with inorganic selenium compounds being significantly more toxic than organic counterparts (15, 16). Moreover, in humans, rare cases of chronic Se-excess have been associated with elevated incidences of amyotrophic lateral sclerosis (17–19). Additionally, elevated selenium intake has been linked to higher incidences of type 2 diabetes (20), as have heightened levels of the selenium transport protein, selenoprotein P (21).

Whereas, the influence of Se has been extensively studied in many contexts, the developmental *in vivo* effects of chronic Se-deficiency and Se-excess upon measures of neurobehavior and energy metabolism have not been comprehensively characterized. Thus, we performed an expansive assessment of various behavioral and metabolic indices in young adult mice receiving dietary supplementation at levels corresponding to Se-deficient, Se-supplemented, and Se-excess upon weaning.

MATERIALS AND METHODS

Animals

All experiments were conducted on male C57BL/6J mice purchased from Jackson labs at 3–4 weeks of age. Mice were maintained on a 12-h light/dark cycle and provided *ad libitum* food and water access. Procedures and experimental protocols were approved by the University of Hawaii's Institutional Animal Care and Use Committee. All efforts were made to minimize animal discomfort and number of animals used.

Diet

Upon arrival at the University of Hawaii Animal Facility, mice were allocated into three groups, representing conditions of Se-deficiency, Se-supplementation, and Se-excess. All mice were administered Se-deficient laboratory chow (∼0.08 ppm Se) (Research Diets, D19101Y), for which casein is the main source of both protein and Se, and the predominant Se species are organic. The Se-supplemented and Se-excess groups received sodium selenite in the drinking water at doses of 10 μM and 100 μM, respectively. Hundred μM sodium selenite corresponds to ∼ 8 ppm elemental Se, a dosage reported to induce mortality in rats (22) and elicit clinical symptoms in humans (23).

Experimental Design

Mice were group-housed until 10 weeks of age and then single-housed 3 days prior to onset of behavioral experiments. Spatial learning was assessed on the Barnes maze at 10–12 weeks of age, followed by metabolic phenotyping at 14–16 weeks. Motor coordination was periodically examined at 8, 12, and 16 weeks of age. Testing for acoustic startle/prepulse inhibition was performed last, at 17–18 weeks, as this procedure involves loud auditory stimuli and could potentially confound other behavioral procedures. At 20 weeks of age, mice were euthanized via CO_2 asphyxiation for collection of fresh tissue or deeply anesthetized (1.2% Avertin; 0.7 ml/mouse) and perfused with 4% paraformaldehyde for immunohistology. Blood was also collected upon sacrifice, and in non-perfused mice, fat depots for gonadal and inguinal white adipose tissue were collected and weighed. Brains from non-perfused mice were split along the sagittal plane, with one hemisphere used for Se analysis and the other hemisphere allocated for biochemical assays.

Barnes Maze Test

Spatial learning was assessed using the Barnes maze (TSE Systems) as described previously (24). In brief, the maze consists of a white circular board containing 40 equally spaced holes, with one hole leading to an escape tunnel. Mice were trained to find the escape tunnel, which remained at a fixed location relative to spatial cues for the duration of training. Training consisted of two trials daily (3 min max per trial) for 10 days, with the starting location varying pseudorandomly among the four quadrants. If a mouse failed to find the escape tunnel within the 3 min trial period, it was placed in the escape tunnel by the researcher and allowed to stay there for 15 s. For each training trial, the latency to locate the escape tunnel and the number of incorrect holes checked (errors) before locating the escape tunnel were recorded. For analysis purposes, data were grouped into trial blocks, which consisted of 4 trials, with each trial administered from a distinct quadrant.

Rotarod Test

Starting speed for the Rotarod was 4 rpm and increased to 40 rpm over a 5 min period. The latency to fall off the rod was measured for each trial and the best score for each mouse was used for statistical analysis.

Acoustic Startle and Prepulse Inhibition

Mice were placed in the startle chamber (Responder-X, Columbus Instruments, Columbus, OH) and allowed a 5-min acclimation period with the background noise (70 dB) continually present. Following acclimation, two blocks of trials were administered to assess the acoustic startle response and prepulse inhibition, respectively, as described previously (25).

Glycemic Control Testing

Glucose tolerance was assessed by administering a glucose injection of 1 mg/g of body weight to animals that were fasted overnight. Tail blood was collected at time points 0, 30, 60, 120, and 180 min after injection and glucose levels were determined using strips and a glucometer (OneTouch Ultra, Lifescan).

Lipid Droplet Analysis of Brown Adipose Tissue (BAT)

BAT was collected from perfused animals ($n = 4$ per group), embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Bright field images were taken at 20× magnification and imported into FIJI for image analysis. Images were thresholded, and droplets were measured using the

"Analyze Particles" feature of FIJI. For each subject, 500–700 lipid droplets were measured.

Metabolic Chambers

Locomotion, respiratory metabolism, and ingestive behavior were measured using the PanLab Oxylet*Pro*^{TM} System (Harvard Apparatus, Barcelona, Spain) according to the manufacturer's instructions. Mice were placed in individual chambers, with fresh bedding, food, and water, and allowed to acclimate for 24 h, followed by 48 h of data collection. Cage air was sampled for 7 min periods every 35 min to measure oxygen and carbon dioxide concentrations. Data were collected and analyzed with Panlab METABOLISM software (Vídenská, Prague, Czech Republic).

Protein Extraction and Immunoblotting

Frozen tissues were lysed by sonication in CelLytic MT buffer (Sigma-Aldrich) containing protease inhibitors (Calbiochem) and centrifuged at 14,000 *g* for 10 min at 4°C. Supernatants were collected and the protein concentrations were measured using the Bradford assay. For western blotting, 40 μg samples of total protein were separated on 4–20% SDS-PAGE gradient gels (Bio-Rad), transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore), and probed for 2 h at room temperature with specific 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). Relative protein levels were determined by dividing the optical density of the band representing the protein of interest by that of the loading control (β-actin).

Antibodies

Primary antibodies used for Western blotting were as follows: goat anti-GPX1 (1:500; R&D Systems, AF3798), mouse anti-SELENBP1 (1:1,000, MBL, M061-3), rabbit anti-TXNRD2 (1:1,000; Invitrogen, LF-PA0024), and rabbit anti-β-actin (1:5,000; Cell Signaling, 4970S).

Leptin ELISA

Serum leptin levels were measured using a commercially available solid-phase sandwich ELISA kit (Invitrogen) according to the manufacturer's instructions.

Se Analysis

Se was measured using a modification of the fluorometric assay of Koh and Benson (26) and Sheehan and Gao (27). Tissue was predigested in 6 ml nitric acid at 150–300°C for 2 h. Hundred μl predigested tissue, serum, or Se standard (Millipore Sigma, 89598) was then digested with 0.5 ml perchloric:nitric acid (1:4) at 197° for 1.5 h. As samples cooled to 150°C, 0.5 ml hydrochloric acid (HCl) was added and samples were maintained at 130–150°C for 30 min. Next, 2 ml 0.1 M EDTA, 0.5 ml 2,3 diaminonaphthalene (0.1% w/v in 0.1 M HCl), and 3 ml cyclohexane were added, followed by incubation at 60°C for 30 min. Fluorescence was measured in a Perkin-Elmer LS 55 fluorometer and concentrations determined via comparison to a standard curve.

Glutathione Peroxidase Activity Assay

Soluble proteins were extracted as described above and normalized to a concentration of 4 mg/ml. Glutathione peroxidase activity was measured as the reduction rate of cumene hydroperoxide catalyzed by the samples upon oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) using a commercially available kit (Cayman Chemical). A unit of activity was defined as the consumption of 1 μmol of NADPH per min, calculated from the expression $(V_{max}$ X $V_t/V_s)/(0.0062$ X D), using 0.0062 μM^{-1} cm^{-1} as the extinction coefficient for NADPH at 340 nm.

Statistical Analysis

Data were analyzed and plotted using Prism software (GraphPad). Statistical tests varied according to the experiment and are indicated in the text and/or figure legends. To compare multiple groups, ANOVAs and Tukey's *post-hoc* test were utilized. All results are represented as mean ± standard error of the mean (SEM).

RESULTS

For this study, newly weaned male mice were allocated into three groups devised to represent conditions of Se-deficiency (Se-def), Se-supplementation (Se-sup), and Se-excess (Se-exc). All mice were fed Se-deficient laboratory chow (∼0.08 ppm Se), with the Se-sup and Se-exc groups receiving additional Se supplementation in their drinking water at doses of 10 μM and 100 μM, respectively. As anticipated, we observed no differences among groups for food intake (**Figure 1A**), although water consumption did vary (**Figure 1B**) [$F_{(2, 17)} = 4.918$, $p = 0.0206$], with significant differences between the Se-def and Se-exc groups ($p = 0.018$). We also calculated Se intake based on water and food consumption, and mean values corresponded to 0.26, 2.14, and 13.94 μg/days for the Se-def, Se-sup, and Se-exc groups, respectively (**Figure 1C**).

Upon sacrifice, tissue was harvested for determination of Se content and additional molecular analyses. For kidney [$F_{(2, 9)} = 78.91$, $p < 0.0001$], liver [$F_{(2, 9)} = 100.5$, $p < 0.0001$], and serum samples [$F_{(2, 9)} = 8.578$, $p = 0.0082$], Se levels differed among groups in a dose-dependent manner, whereas in brain [$F_{(2, 8)} = 0.7685$, $p = 0.4951$] and testes [$F_{(2, 9)} = 3.430$, $p = 0.0781$], levels were comparable (**Figure 1D**). Parallel analyses of glutathione peroxidase (GPx) activity were conducted on liver, serum, and brain samples. Surprisingly, liver GPx activity [$F_{(2, 9)} = 2.256$, $p = 0.1606$] was similar between groups, whereas serum GPx activity [$F_{(2, 9)} = 0.5429$, $p = 0.5937$] showed similar non-significant trends as observed for Se analysis (**Figure 1E**). For brain, we detected a significant main effect of Se group upon GPx activity [$F_{(2, 9)} = 4.263$, $p = 0.0498$], with differences between the Se-def and Se-sup groups attaining significance ($p = 0.0441$). Western blotting was also performed on liver and brain samples to assess various markers of Se status. We probed for GPX1 and TXNRD2, two abundant selenoproteins known to be responsive and non-responsive to alterations in Se supply (28), respectively, and the selenium binding protein (SELENBP1), a putative factor protecting against Se-toxicity (29). For liver samples, levels of

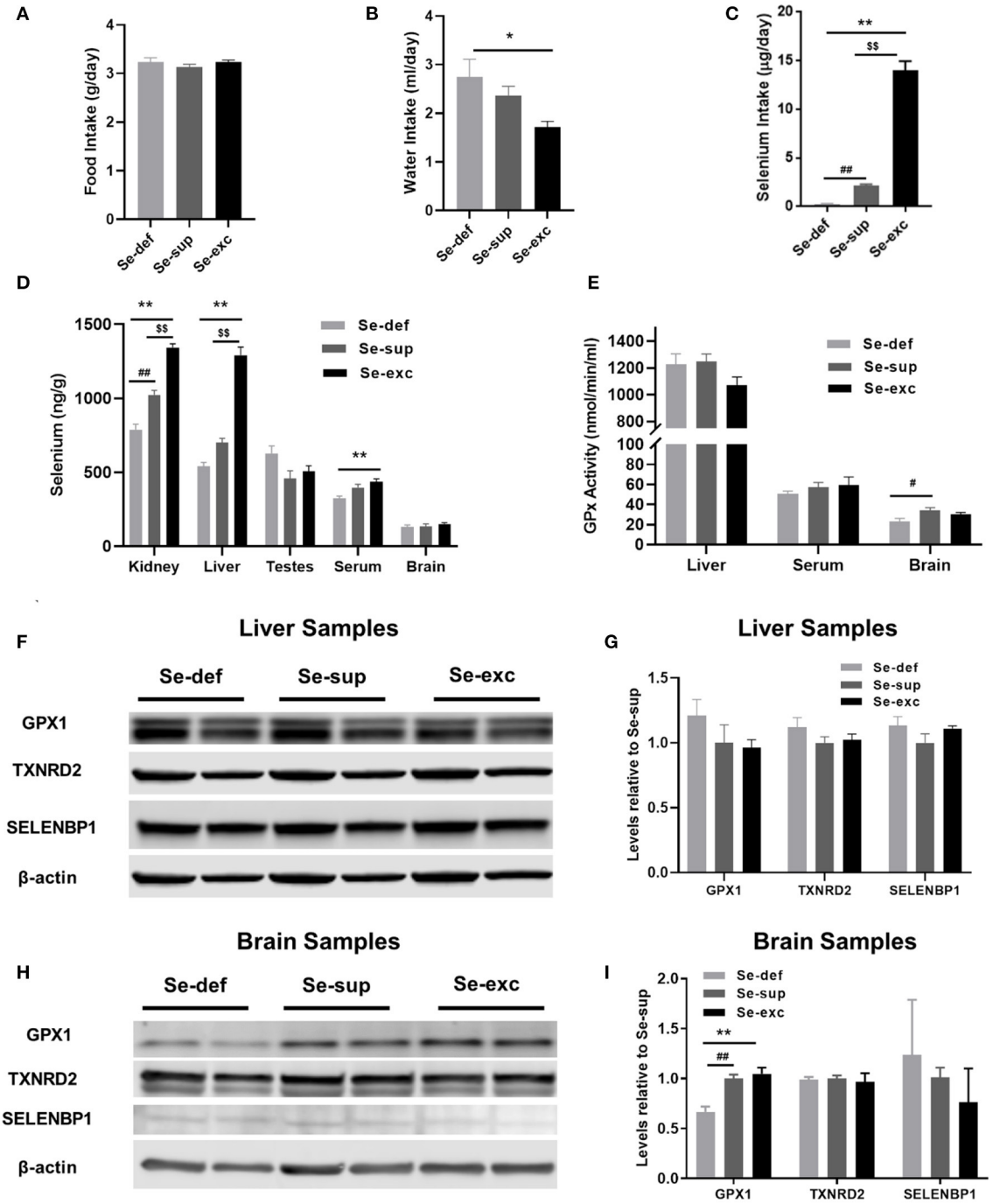

FIGURE 1 | Assessment of varying Se supplementation on organ-specific Se content, glutathione peroxidase activity, and selenoprotein levels. **(A)** Mean (±SEM) daily food intake. **(B)** Mean (±SEM) daily water consumption. **(C)** Mean (±SEM) daily selenium intake (n = 6–7 per group). **(D)** Mean (±SEM) selenium content in kidney, liver, testes, serum, and brain (n = 3–4 per group). **(E)** Mean (±SEM) GPX activity in liver, serum, and brain (n = 4 – 6). **(F–I)** Protein levels of GPX1, TXNRD2, and SELENBP1 in liver **(F,G)** and brain **(H,I)** (n = 4). $^{\$\$}p < 0.01$ between Se-exc and Se-sup groups; $^{*}p < 0.05$ between Se-exc and Se-def groups; $^{**}p < 0.01$ between Se-exc and Se-def groups; $^{\#}p < 0.05$ between Se-def and Se-sup groups; $^{\#\#}p < 0.01$ between Se-def and Se-sup groups.

GPX1, TXNRD2, and SELENBP1 were not impacted by Se group (**Figures 1F,G**). Brain levels of TXNRD2 and SELENBP1 were comparable between groups, but we did observe altered levels of GPX1 [$F_{(2, 9)} = 15.67$, $p = 0.0012$], as levels were significantly reduced in the Se-def group (vs Se-sup: $p = 0.0038$; vs. Se-exc: $p = 0.0016$) (**Figures 1H,I**).

Prior to harvesting of tissue, mice were subjected to a battery of neurobehavioral and metabolic tests. Cognition was evaluated using the Barnes maze, a widely utilized paradigm for spatial learning in rodents. Mice were trained to find a hidden escape tunnel located beneath one of 40 holes on the periphery of the circular maze. As anticipated, we observed a main effect of time on spatial learning, as indicated by less primary errors [$F_{(5, 100)} = 88.6$, $p < 0.001$] and a faster primary latency ($F_{(5, 100)} = 66.7$, $p < 0.0001$] when locating the escape tunnel (**Figures 2A,B**). We also detected a main effect of Se group upon the number of primary errors [$F_{(2, 25)} = 4.907$, $p = 0.0159$], but not upon the primary latency [$F_{(2, 25)} = 1.44$, $p = 0.2560$]. For both primary errors [$F_{(8, 100)} = 1.697$, $p = 0.1083$] and primary latency [$F_{(8, 100)} = 1.015$, $p = 0.4295$], the time × Se group interaction was non-significant. *Post-hoc* analyses revealed higher levels of primary errors in the Se-def group during trial block one (vs. Se-exc: $p = 0.0151$) and two (vs Se-sup: $p = 0.0008$; vs. Se-exc: $p = 0.0233$). Likewise, primary latencies were significantly higher in the Se-def group during trial block 2 (vs Se-sup: $p = 0.0146$; vs. Se-exc: $p = 0.0144$). No significant differences were observed between groups for these measures during the remaining trial blocks nor during a probe trial conducted after trial block 5 (data not shown). As a whole, these results indicate that spatial learning is impaired by Se-deficiency.

Motor coordination, as determined by the latency to fall off a rotating rod of increasing speed, was examined at 8, 12, and 16 weeks of age (**Figure 2C**). Two-way ANOVA analyses revealed a main effect of time [$F_{(2, 50)} = 7.826$, $p = 0.0011$) and a significant time x Se group interaction effect [$F_{(4, 50)} = 2.779$, $p = 0.0367$], whereas the influence of Se group was non-significant [$F_{(2, 25)} = 1.208$, $p = 0.3156$]. In our initial test at 8 weeks of age, we observed differences between the Se-sup and Se-exc groups, with Se-exc mice performing significantly worse ($p = 0.0252$). Surprisingly, motor coordination improved over time in the Se-exc group, whereas performance declined in both the Se-def and Se-sup groups.

To assess sensorimotor gating, mice were tested for acoustic startle reactivity and prepulse inhibition. For acoustic startle (**Figure 2D**), we observed a main effect for stimulus intensity [$F_{(6, 150)} = 53.31$, $p < 0.0001$), whereas both Se group [$F_{(2, 25)} = 2.241$, $p = 0.1273$] and the stimulus intensity × Se group interaction [$F_{(12, 150)} = 1.610$, $p = 0.0942$] were not significant. Across the vast majority of stimulus intensities, startle magnitude was most pronounced in the Se-exc group, with statistically significant differences detected at 95 dB (vs. Se-sup: $p = 0.0410$) and 110 dB (vs. Se-def: $p = 0.0025$). In testing for prepulse inhibition (**Figure 2E**), a main effect of prepulse intensity [$F_{(2, 50)} = 16.07$, $p < 0.0001$] was found, while the effects of Se group [$F_{(2, 25)} = 1.062$, $p = 0.3608$] and the prepulse intensity × Se group interaction [$F_{(4, 50)} = 2.185$, $p = 0.0841$] failed to reach significance. Moreover, at the highest prepulse intensity (16 dB), the Se-def group exhibited significantly reduced inhibition relative to the Se-exc group ($p = 0.0361$).

Mice were also tested for glycemic control and body weight was regularly monitored. For glucose tolerance testing (**Figure 3A**), a main effect of time [$F_{(4, 76)} = 63.18$, $p < 0.0001$] and a significant time x Se group interaction effect [$F_{(8, 76)} = 2.454$, $p = 0.0203$] was detected, whereas the effect of Se group was non-significant [$F_{(2, 19)} = 1.680$, $p = 0.2129$]. *Post-hoc* tests revealed significantly elevated blood glucose levels 120 min after glucose injection in the Se-def group relative to the Se-exc group ($p = 0.0148$). With respect to body weight, two-way ANOVA analysis revealed a main effect of time [$F_{(8, 200)} = 316.1$, $p < 0.0001$], with non-significant effects observed for Se group [$F_{(2, 25)} = 2.307$, $p = 0.1203$] and the time × Se group interaction [$F_{(16, 200)} = 0.4277$, $p = 0.9740$]. Levels gradually diverged over time between the Se-def and Se-exc groups, with differences reaching significance at 20 wks (**Figure 3B**) ($p = 0.0443$). Upon sacrifice, we also found that relative levels of inguinal white adipose tissue (iWAT) differed between groups (**Figure 3C**) [$F_{(2, 9)} = 6.227$, $p = 0.0201$], with statistically significant differences between the Se-def and Se-exc groups ($p = 0.0165$). Similar non-significant trends were also observed for gonadal white adipose tissue (gWAT) (**Figure 3D**) [$F_{(2, 9)} = 2.119$, $p = 0.1762$] and serum leptin (**Figure 3E**) [$F_{(2, 21)} = 2.563$, $p = 0.1009$]. Finally, average lipid droplet size in brown adipose tissue (BAT) significantly differed between groups (**Figures 3F,G**) [$F_{(2, 7,750)} = 77.77$, $p < 0.0001$], as droplets were larger in the Se-def group ($p < 0.0001$).

At 14–16 weeks of age, mice were placed in metabolic chambers for 48-hrs to evaluate activity, respiratory metabolism, and ingestive behavior. For locomotion (**Figures 4A,B**), two-way ANOVA analysis detected main effects for both light cycle [$F_{(1, 17)} = 46.81$, $p < 0.0001$] and Se-group [$F_{(2, 17)} = 7.126$, $p = 0.0057$], in conjunction with a non-significant light cycle x Se group interaction effect [$F_{(2, 17)} = 0.809$, $p = 0.4617$]. During both the light ($p = 0.0164$) and dark cycles ($p = 0.0018$), the Se-exc group exhibited elevated locomotion relative to the Se-sup group. For measures of energy expenditure (**Figure 4C**) [EE: $F_{(1, 17)} = 1{,}238$, $p < 0.0001$] and the respiratory quotient (**Figure 4D**) [RQ: $F_{(1, 17)} = 140.2$, $p < 0.0001$], we also observed a main effect for the light cycle, but not for Se group [EE: $F_{(2, 17)} = 0.5002$, $p = 0.6151$; RQ: $F_{(2, 17)} = 3.126$, $p = 0.0698$]. Moreover, we also detected a significant light cycle x Se group interaction effect for energy expenditure [EE: $F_{(2, 17)} = 4.133$, $p = 0.0344$], but not for the respiratory quotient [RQ: $F_{(2, 17)} = 1.212$, $p = 0.3222$].

DISCUSSION

In summary, these results detail the negative consequences of juvenile Se-deficiency upon measures of behavior and metabolism in early adulthood. Se-deficient mice displayed delayed learning and altered sensorimotor gating, and these deficits coincided with reduced GPx activity in brain. Moreover, Se-deficiency also resulted in impaired glycemic control, elevated body weight, and increased adiposity. Finally, Se-excess, at levels known to be toxic to humans, was surprisingly well-tolerated in mice and exerted beneficial effects on energy metabolism.

Our study corroborates prior findings that Se-deficiency hinders spatial learning (30, 31) and, to the best of our knowledge, represents the first association of Se-deficiency with impairments in sensorimotor gating. Deficits in cognition and sensorimotor

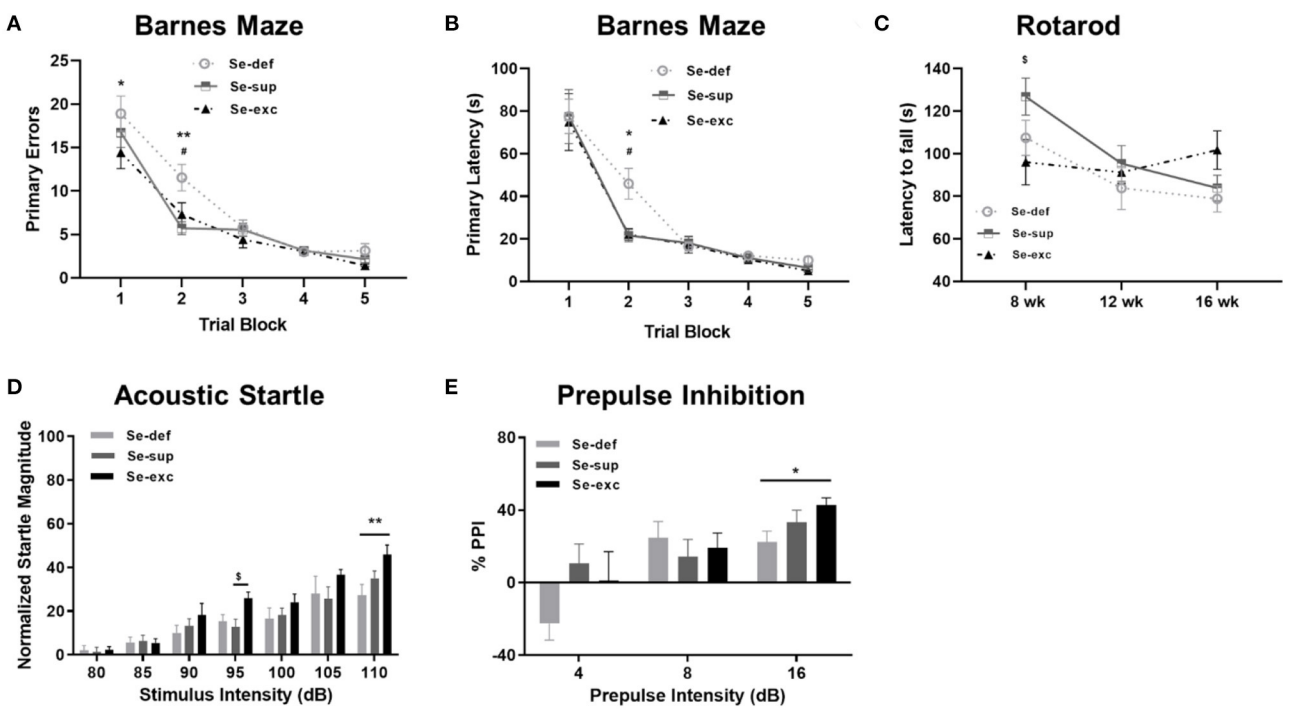

FIGURE 2 | Se-deficient mice exhibit deficits in cognition and sensorimotor gating. **(A)** Mean (±SEM) number of incorrect holes checked before locating the escape tunnel during Barnes maze training. **(B)** Mean (±SEM) latency to locate the escape tunnel during maze training. **(C)** Mean (±SEM) latency to fall off the Rotarod at 8, 12, and 16 weeks of age. **(D)** Mean (±SEM) normalized startle magnitude as a function of acoustic startle intensity. **(E)** Mean (±SEM) percentage of prepulse inhibition as a function of prepulse intensity above the background level (70 dB). $^{\$}p < 0.05$ between Se-exc and Se-sup groups; $^{*}p < 0.05$ between Se-exc and Se-def groups; $^{**}p < 0.01$ between Se-exc and Se-def groups; $^{\#}p < 0.05$ between Se-def and Se-sup groups ($n = 9$–10 animals per group for all experiments).

gating are hallmarks of many neurodevelopmental disorders, including schizophrenia and autism. For both schizophrenia (11, 32, 33) and autism (12, 34, 35), reduced Se levels have been chronicled in the literature, albeit there are many exceptions (36–38), and it is unclear whether this represents a cause or consequence of these conditions. Of particular significance to our results is a recent report examining the Se status of 287 Polish children, which were divided into four groups, corresponding to: (1) autism spectrum disorder (ASD) with obesity, (2) ASD without obesity, (3) non-ASD with obesity, and (4) non-ASD without obesity (12). Observed Se levels were lowest in ASD patients with obesity and highest in non-ASD patients without obesity, with differences between groups being highly significant ($p < 0.001$) for serum, urine, toenail samples. Moreover, across groups, Se levels were inversely correlated with body mass index ($p < 0.001$) for all sample types.

The influence of Se supplementation upon energy metabolism is hotly debated and nuanced in the existing literature. An unanticipated corollary of the Nutritional Prevention of Cancer (NPC) trial was the observation that Se-supplementation (200 μg daily as Se-yeast) increased risk of type 2 diabetes for participants with baseline plasma Se levels within the upper tertile (20). Since these findings were documented, excess Se supplementation has been shown to adversely impact insulin signaling in multiple rodent models (39, 40). In contrast, reduced serum Se levels have been observed in morbidly obese patients (41), and supranutritional Se supplementation (240 μg/day) in the form of selenomethionine (SeMet) was recently shown to decrease both fat mass and circulating leptin levels in a 3-months dietary intervention study of obese individuals (42). Of potential relevance, we previously reported increased adiposity and elevated leptin levels in mice lacking SELENOM (43), an ER-resident selenoprotein that is highly expressed in brain and regulated by Se levels. Further studies showed that leptin upregulates SELENOM in hypothalamic neurons and that SELENOM, in turn, promotes leptin signaling (44). More recently, supranutrional Se supplementation (2.25 ppm SeMet in chow) was found to facilitate selenocysteine incorporation at sites canonically encoding cysteine, promote thermogenesis, and protect against diet-induced obesity (45).

One unexpected outcome of this study was the beneficial influence of Se at a dosage (8 ppm in water) originally hypothesized to elicit toxic effects. We chose to use chow that was mildly Se-deficient and provide further Se supplementation in the drinking water as selenite to the Se-sup and Se-exc groups. Inorganic Se species (selenite, selenate) are less readily absorbed by the intestine than organic counterparts (SeMet) (46–48), and are also significantly more toxic. For instance, the toxicity of selenite was found to be 53-fold greater than that of SeMet when administered intracerebroventricularly to rats (15). With specific regard to supplementation of inorganic Se species in drinking water, increased mortality was previously reported at

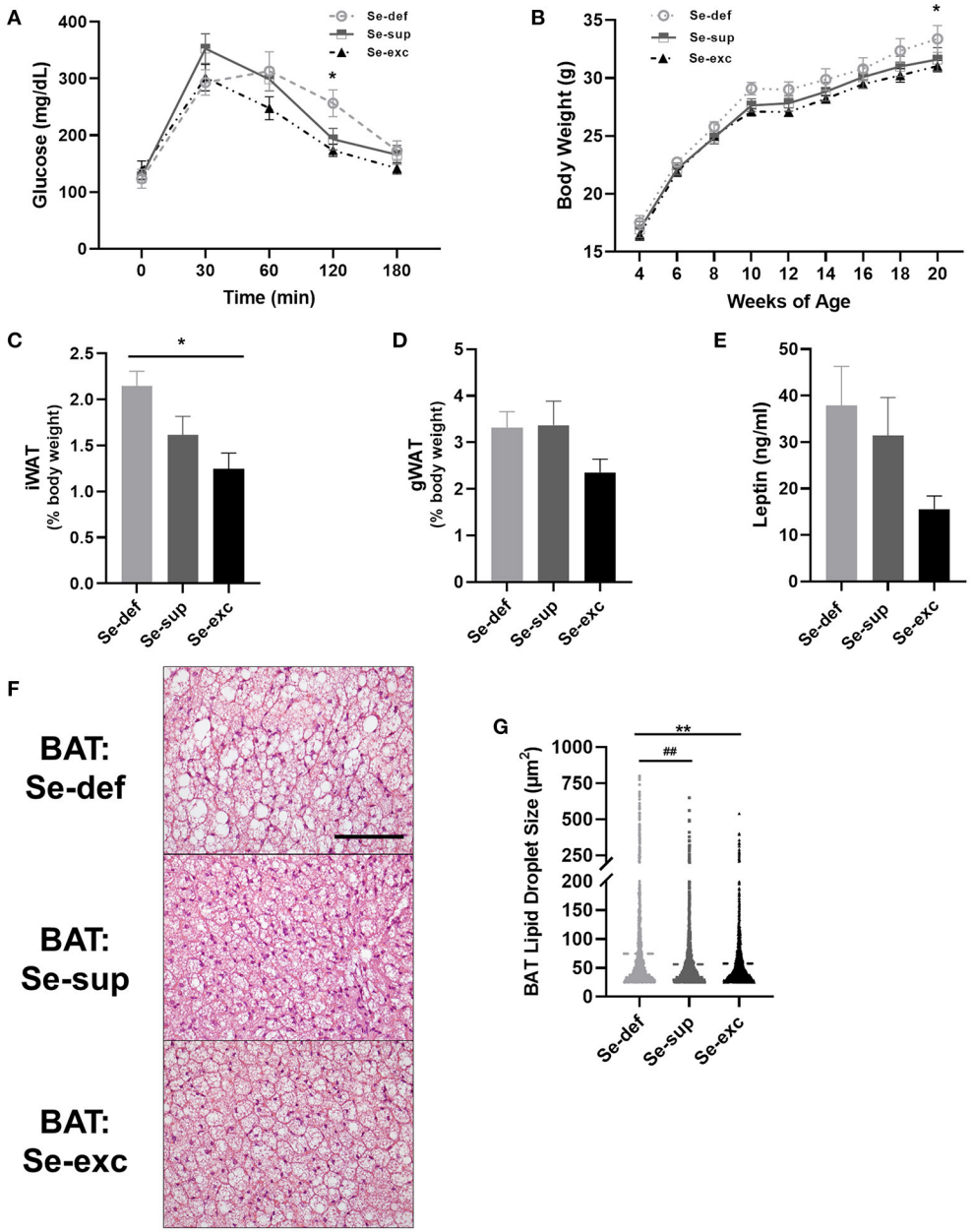

FIGURE 3 | Impaired glucose tolerance and increased adiposity in Se-deficient mice. **(A)** Mean (±SEM) blood glucose levels during glucose tolerance testing ($n = 7$–8 per group). **(B)** Mean (±SEM) body weight from 4 to 20 weeks of age ($n = 9$–10). **(C)** Mean (±SEM) inguinal white adipose tissue ($n = 4$). **(D)** Mean (±SEM) gonadal white adipose tissue relative to total body weight ($n = 4$). **(E)** Mean (±SEM) serum leptin levels ($n = 7$–9). **(F)** Representative images of brown adipose tissue (BAT). **(G)** Scatter plot of BAT lipid droplet size ($n = 4$). Scale bar = 100 μm, $^{*}p < 0.05$ between Se-exc and Se-def groups, $^{**}p < 0.01$ between Se-exc and Se-def groups; $^{\#\#}p < 0.01$ between Se-def and Se-sup groups.

levels >6 ppm, although lower Se dosages (2–3 ppm) did lead to decreased body weights (22). Similarly, chow containing Se at >5 ppm, has been shown to adversely impact growth and mortality in rodents (49) and pigs (50), with effects being more severe when selenite was the predominant Se species. Although relatively rare in humans, Se intoxication leads to loss of hair and nails, skin lesions, and nervous system abnormalities. A case study of Se toxicity in the Enshi district of China reported neurological defects in 18 of 22 subjects displaying signs of selenosis, and symptoms included hyperreflexia, convulsions, motor weakness, and hemiplegia (51). Moreover, blood Se levels in affected patients were observed to be roughly 100 times greater than subjects receiving a Se-adequate diet. Chronic Se overexposure has also been associated with an elevated risk

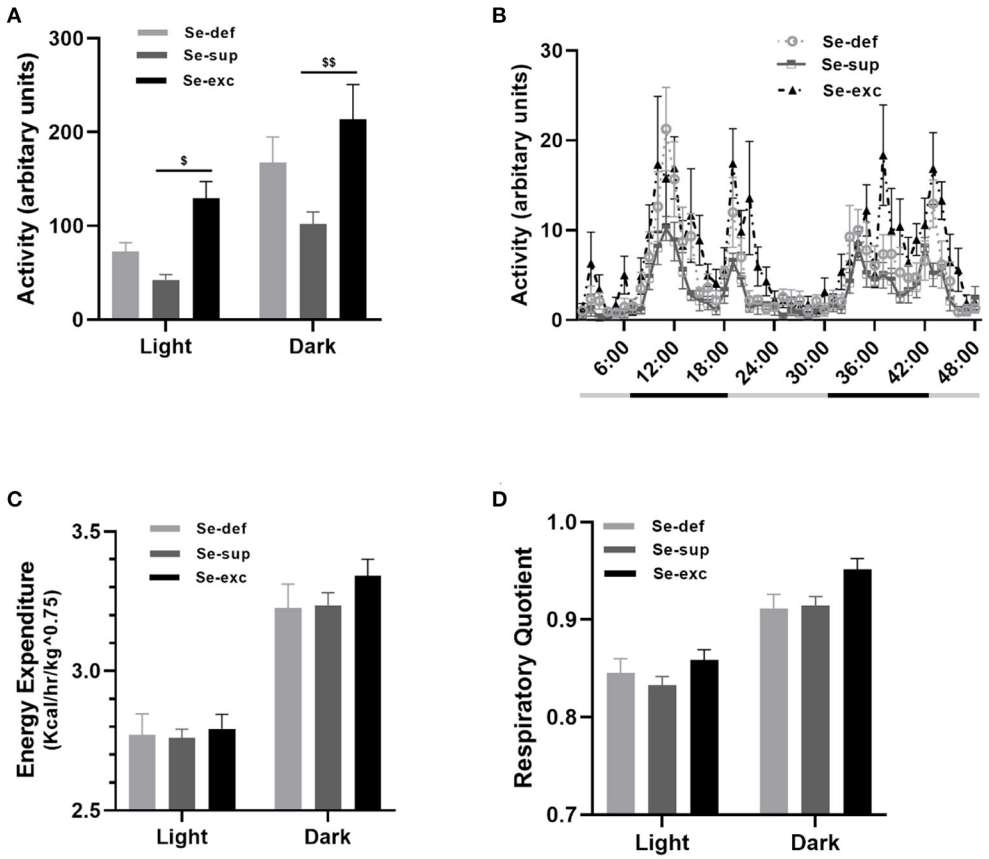

FIGURE 4 | Influence of varying Se supplementation on locomotion and respiratory metabolism. **(A)** Mean (±SEM) locomotor activity during the light and dark cycles. **(B)** 48-h time course of locomotor activity. **(C)** Mean (±SEM) energy expenditure during the light and dark cycles. **(D)** Mean (±SEM) respiratory quotient during the light and dark cycles. $^{\$}p < 0.05$ between Se-exc and Se-sup groups; $^{\$\$}p < 0.01$ between Se-exc and Se-sup groups; ($n = 6$–7 animals per group for all experiments).

of neurodegenerative disease, specifically amyotrophic lateral sclerosis (ALS). This linkage was first noted in 1977, when a cluster of ALS cases was reported in a seleniferous area of South Dakota (19) and further substantiated by increased incidences of ALS in an Italian population chronically exposed (1974–1988) to drinking water containing high levels of selenate (18). Of further significance, elevated levels of selenite have been reported in the cerebrospinal fluid (CSF) of newly diagnosed ALS patients (52).

Another unanticipated finding was that Se supplementation modulated GPx activity to a greater extent in brain than liver. Brain Se levels are typically lower than other organs and blood (53), with Se homeostasis in the nervous system being tightly regulated by the blood-brain barrier (BBB) (54). Se transport to brain is regulated by endothelial cell-mediated uptake of SELENOP via the lipoprotein-related receptor, ApoER2, at the BBB (54, 55). SELENOP is also expressed in astrocytes (56–58), especially those lining the BBB, and astrocyte-derived SELENOP is speculated to supply ApoER2-expressing neurons with Se within the parenchyma (59). In cases of severe Se-deficiency, it is known that the brain and testes preferentially retain Se at the expense of other organs, and this phenomenon is dependent upon SELENOP and ApoER2 (55, 60–62). It should be duly noted that our Se-deficient chow contained 0.08 ppm Se, several-fold higher than that of many Se deprivation studies, but still well below the 0.15 ppm minimum recommended for rodent diets by the AIN (63). Given that liver and kidney represent the primary sites of Se metabolism and excretion (64), respectively, the fact that supplementation most impacted Se content in these tissues was expected. The effect of supplementation on serum Se was less robust, suggesting that most Se was converted to excretory metabolites in liver, with a small fraction being incorporated into SELENOP. Moreover, it appears that our Se-deficient diet did not affect liver GPx activity, in line with prior findings by Sunde and colleagues showing that hepatic levels of GPx activity plateau when dietary Se is 0.09 ppm or greater (65). Furthermore, prior evidence suggests that supplementation at levels similar to our study can significantly impact brain GPx activity. For example, Whanger and colleagues reported that increasing dietary Se content from 0.1 to 4 ppm raised GPX activity by 32 and 77% in cortex and cerebellum, respectively (66).

It is imperative to note this study has several caveats that merit consideration. First, to reduce cost and animal usage, only

male mice were used. Sex-specific differences in the biological effects of Se are well-documented in the literature (67–69), with males typically being more adversely impacted by deviations in Se intake. Second, experiments were conducted on young adult mice (3–5 months) and the possibility that long-term Se overexposure elicits neurodegenerative effects at later time points cannot be ruled out. Finally, given that our dietary intervention began shortly after weaning, it is probable that Se supplementation triggered developmental epigenetic adaptations to cope with Se-excess. Interestingly, Se-excess mice performed significantly worse in the initial rotarod test, but their performance improved over time, while that of the other two groups (Se-def, Se-sup) declined. It is quite possible that providing adult mice with Se at our chosen excessive dose may elicit detrimental toxic effects not observed in juveniles.

Nevertheless, these results detail the adverse effects of mild Se-deficiency and suggest that juvenile Se status is critical for optimal neurodevelopment. These findings may have important implications for future prevention and treatment of neurodevelopmental disorders where redox imbalance is a key characteristic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Hawaii's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

VK and MP designed the experiments. VK, AS, DT, CC, JP, and MP performed research. CW contributed reagents/analytic tools. MP analyzed data and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Therapeutic Potential of Selenium in Glioblastoma

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Little progress has been made in the long-term management of malignant brain tumors, leaving patients with glioblastoma, unfortunately, with a fatal prognosis. Glioblastoma remains the most aggressive primary brain cancer in adults. Similar to other cancers, glioblastoma undergoes a cellular metabolic reprogramming to form an oxidative tumor microenvironment, thereby fostering proliferation, angiogenesis and tumor cell survival. Latest investigations revealed that micronutrients, such as selenium, may have positive effects in glioblastoma treatment, providing promising chances regarding the current limitations in surgical treatment and radiochemotherapy outcomes. Selenium is an essential micronutrient with anti-oxidative and anti-cancer properties. There is additional evidence of Se deficiency in patients suffering from brain malignancies, which increases its importance as a therapeutic option for glioblastoma therapy. It is well known that selenium, through selenoproteins, modulates metabolic pathways and regulates redox homeostasis. Therefore, selenium impacts on the interaction in the tumor microenvironment between tumor cells, tumor-associated cells and immune cells. In this review we take a closer look at the current knowledge about the potential of selenium on glioblastoma, by focusing on brain edema, glioma-related angiogenesis, and cells in tumor microenvironment such as glioma-associated microglia/macrophages.

Keywords: selenium, glioblastoma, glutamate, SLC7A11, microglia

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INTRODUCTION

Glioblastoma is by far the most common occurring malignant primary brain tumor in adults, affecting approximately 15% of all primary brain tumors diagnosed annually in the United States (Ostrom et al., 2019) and 20% in Europe (Wohrer et al., 2009; Darlix et al., 2016). Despite the aggressive multimodality strategy, the current prognosis of patients with glioblastoma (WHO Grade IV) is poor, with a median survival time of only 12–15 months (Wen and Kesari, 2008). The aggressive infiltrative growth of malignant glioma cells and the development of tumor angiogenesis are still therapeutic obstacles (Jain et al., 2007). Both, the complex molecular intra- and inter-tumoral heterogeneity of glioblastoma as well as the evidence of glioma stem cells (GSCs) in tumor microenvironment (TME), make a complete surgical resection impossible (Cheng et al., 2013; Soeda et al., 2015). Thus, tumor recurrence is an expected result after high-grade glioma surgery despite maximal and supramaximal resection.

Furthermore, although cell death of all tumor cells was observed in glioblastoma cell lines treated with certain concentrations of the chemotherapeutic agent temozolomide (TMZ, Temcat®, Temodal®, or Temodar®) *in vitro*, the life expectancy of patients with glioblastoma increases by not more than 2.5 months (Stupp et al., 2005). One of the major reasons for this outcome is that this oral alkylating agent has a limited selectivity toward malignant cells. In other words, the very toxic treatments such as radiation and chemotherapy indiscriminately attack all cells, including healthy cells causing extensive cellular damage and cytotoxicity. This gives rise to recurrence of glioblastoma and promotes development of drug resistance in tumor cells. However, in order to develop novel therapeutic strategies to treat these malignant brain tumors successfully, it is indispensable to have a better understanding of why conventional therapies fail to target malignant cells and often result in tumor relapse.

Selenium (Se) is an important nutritional supplement that is becoming more popular in clinical researches. Se is a key component that can be found in vegetables, soil or meat, but it can also be easily obtained as a dietary supplement without prescription. The initial enthusiasm for supplemental Se intake was based on its anti-oxidative functions. Also, Se has been known for many years to have chemo-preventive functions (Yakubov et al., 2014). The use of Se compounds as a therapeutic agent in malignant tumors was first mentioned by the French surgeon Pierre Delbet at the beginning of the last century, describing the death of patients who received lethal overdoses of sodium selenate (Delbet, 1912). That Se might protect against glioblastoma was first suggested in the late 1980s based on observations of reduced serum concentrations of Se in patients suffering from brain malignancies (Philipov and Tzatchev, 1988). Experimental and observational studies demonstrate that a treatment with Se in non-Se-deficient subjects can not only reduce cancer risk, but it can also regulate several molecular processes in tumorigenesis such as a proliferation, redox homeostasis, angiogenesis, brain edema, and the immune system (Streicher et al., 2004; Carlson et al., 2010; Hall et al., 2013; Carlisle et al., 2020).

In the present review, we summarize the current knowledge about the potential of Se on the treatment of malignant brain tumors. Particularly, we focus on brain edema, glioma-related angiogenesis, and cells in TME such as glioma-associated microglia/macrophages.

MATERIAL SEARCH STRATEGY

Scopus and Web of Science were the main tools of systematic literature searching, whereas PubMed was used as an additional database. The primary time period for the review was January 2000 until January 2021. Research articles and reviews were identified using the search terms (title, abstract, and keywords) "selenium" or "selenoprotein" either alone or in combination with cysteine, glutamate and glutamine as well as these combined with the terms "glioblastoma," "glioma," "cancer," "stem cell," "brain edema," "angiogenesis," "energy metabolism," and "microglia." Additional searches were performed for the exact proteins, namely COX-2, GPx4, GLS, MIF, SLC7A11, and SEPHS2. Relevant papers identified by this search were reviewed, and the references therein were further considered for other useful leads. Epidemiological research on Se supplementation, studies on Se speciation and neurodegenerative disease were not within the main focus of the current review as they have been reviewed recently (Weekley and Harris, 2013; Lopes da Silva et al., 2014; Vinceti et al., 2014, 2018; Yakubov et al., 2014; Solovyev, 2015; Collery, 2018; Gandin et al., 2018).

GLIOMA MICROENVIRONMENT AND THE TUMOR ZONE MODEL

The debate over how much to push the limits of surgical resection for malignant gliomas is not a recent controversy. Therefore, one can conclude that the most favorable outcomes of glioma surgery are achieved in cases with supramaximal resection. Nonetheless, one of the founding fathers of modern neurosurgery, Walter Dandy, performed hemispherectomies in 1928 for patients with malignant gliomas and found that these tumors still recurred on the contralateral side despite such extremely aggressive resection (Dandy, 1928). A rationale for an invasive migration of glioma cells may be explained based on a theoretical consideration of the glioma microenvironment, classifying the tumor into at least three zones (Eyupoglu et al., 2013). The borders of each one of these transition zones may show a smooth shift into the next tumor zone (**Figures 1A–C**). The main section of the tumor, tumor zone I (TZ I), comprises the tumor core cells and can easily be spotted as the contrast-enhancing regions on magnetic resonance imaging (MRI) (**Figure 1D**).

Tumor zone II (TZ II) – the peritumoral zone – consists of glioma "transitory cells," due to the fact that these are cells that show some of the histological characteristics of the glioma core cells that can be found in TZ I. TZ II is rated as the most biologically active area of the tumor because, aside from containing glioma core cells, it also contains glioma-associated microglia and endothelial cells (**Figure 1C**). This area displays hypervascularization, which also represents a challenge in adjuvant treatments. Although malignant gliomas show an accumulation of immune cells, these cannot generate an adequate immune response. The extension of TZ II can be observed on MRI as the area of perifocal edema (**Figure 1E**). Tumor zone III (TZ III) contains the so-called "partisan cells" including isolated glioblastoma cells, tumor-initiating (glioma stem) cells, or precursor cells. TZ III, compared to TZ I or TZ II, appears to be clinically silent, which can be challenging for therapy because it mainly consists of brain tissue. However, partisan cells are probably responsible for tumor recurrence following surgery.

Various factors are secreted and released in these zones, triggering tumor expansion and encouraging key mechanisms for tumor cell progression. These factors foster glioblastoma development and can induce tumor angiogenesis, increase the perifocal edema, lead to neural cell death, paralyze immune cells, or stimulate its proliferation and invasion (**Figure 1**; Savaskan et al., 2008; Yakubov et al., 2014; Choi et al., 2015;

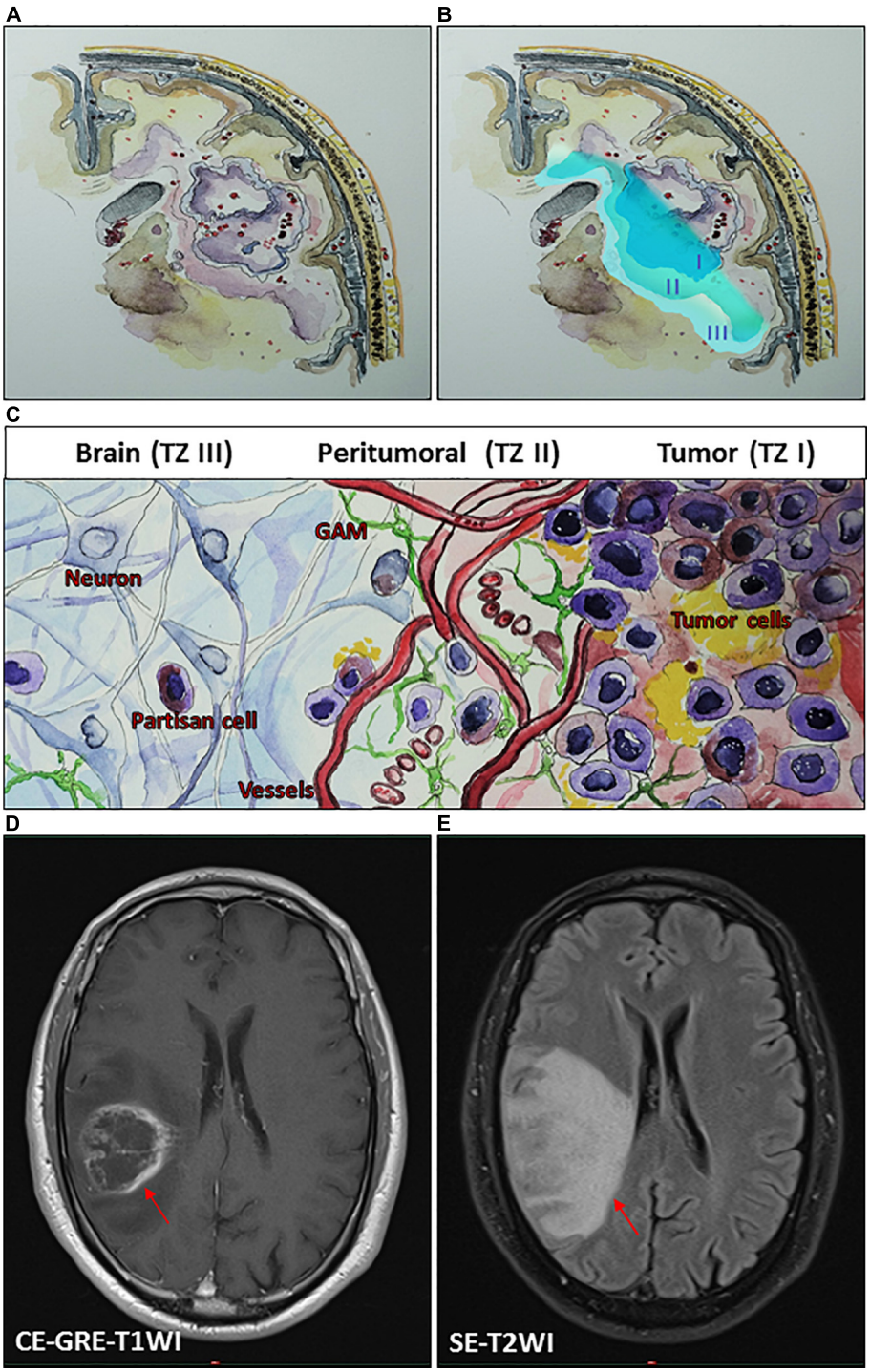

FIGURE 1 | Conceptual schematic illustration of glioma microenvironment classifying the tumor into three heterogeneous tumor zones (TZ I–III). **(A,B)** An illustrative representation of TZ I–III. **(C)** A schematic illustration of glioma microenvironment showing the cellular level of TZ I–III. TZ I comprises the tumor core cells and can be spotted as the contrast-enhancing regions on magnetic resonance imaging (MRI) **(D)**. TZ II, the most biologically active area of the tumor can be observed on MRI as the area of perifocal edema **(E)**, which is characterized by its specific pro-angiogenic microenvironment and the presence of glioma-associated microglia/macrophages (GAM) and transitory cells. TZ III consists mainly of healthy brain tissue and contains isolated partisan cells, which are probably responsible for tumor recurrence following surgery. **(D,E)** Illustrative MRI scans of a patient with a right parietal glioblastoma (WHO grade IV) by using a 1.5 tesla Ingenia scanner (Philips Healthcare, Best, Netherlands). **(D)** A T1-weighted contrast-enhanced gradient-echo imaging (CE-GRE-T1WI), demonstrating the TZ I (arrow). **(E)** A T2-weighted spin-echo imaging (SE-T2WI), showing the TZ II (arrow).

Ghoochani et al., 2016a,b). An ideal surgical approach within malignant gliomas implies an exhaustive resection of TZ I with partial resection of TZ II. A complete resection of TZ II can rarely be reached. However, a supramaximal resection of all tumor zones (TZ I–III) is particularly unattainable. Even though further tumor cell reduction can be achieved with adjuvant radiochemotherapy, some persistent partisan cells will inevitably remain within the brain (Robin et al., 2014). In this scenario, surgery and following radiochemotherapy is iterated, primarily aiming to work against the space occupying consequence of the recurrence. This cycle results in a selection of persistent tumor cells leading to acquired chemoresistant tumors. Due to this repetitive selection, the period time from surgery until recurrence diminishes with every next cycle. These cycle scenarios cause that measures such as surgery or radiochemotherapy are not able to control tumor progression onward.

Therefore, neuro-oncological approaches concerning malignant gliomas are essentially challenging. The current limitations in surgical treatment and radiochemotherapy outcomes encourage researches to look up for better suitable treatments that can promise better achievements.

GLUTAMATE EXCITOTOXICITY AND SELENIUM IN GLIOBLASTOMA

Aside from uncontrolled proliferation and diffuse brain infiltration, neurodegeneration and brain edema represent the feared hallmarks of malignant brain tumors (Wen and Kesari, 2008; Savaskan and Eyupoglu, 2010). Brain edema crucially contributes to the clinical course and outcome of patients with high-grade gliomas (HGGs, WHO grades III and IV) (Carlson et al., 2007). Glioma-induced brain edema is caused by two interdependent mechanisms: HGGs primarily induce abnormal angiogenesis with impaired blood-brain barrier, allowing plasma to enter the interstinal space referred to as vasogenic edema (Wen et al., 2010). Furthermore, HGGs induce neuronal cell death and neurodegeneration by which cytotoxic brain edema can be formed inducing neurological deficits and intractable seizures (Savaskan et al., 2008; Pace et al., 2013).

The neurotoxic amounts of the amino acid glutamate is thought to play a major role in TME interactions leading to the development of a cytotoxic edema in peritumoral regions (TZ II) (Takano et al., 2001; Savaskan et al., 2008; Marcus et al., 2010; Choi et al., 2015), supporting the role of glutamate in glioma cell infiltration also into the TZ III (Marcus et al., 2010; Corbetta et al., 2019). Nevertheless, glioma stem cells (GSCs) were reported to exhibit high drug and radioresistance with migratory potential, and the enriched proportion of GSCs aggravates the tumor (Bao et al., 2006; Chen et al., 2012; Nusblat et al., 2020). A relevant mechanism is represented by the system X_C^- transporter (**Figure 2**), particularly, of its light chain xCT (SLC7A11). This is instrumental in glioblastoma release of excitotoxic concentrations of glutamate into extracellular milieu, which exchanges intracellular glutamate for extracellular cystine (Choi et al., 2015). Intracellularly, cystine is readily converted to cysteine, the rate-limiting precursor for glutathione (GSH) synthesis, resulting in an increased proliferation and GSH-related drug resistance in various cancers (Doxsee et al., 2007; Narang et al., 2007; Polewski et al., 2017; Guo et al., 2020). Also, there are indications that xCT is implicated in tumor-associated epileptic events and predicts poor survival in patients with glioblastoma (Buckingham et al., 2011; Yuen et al., 2012; Robert et al., 2015). In addition, it was recognized that glutamate/glutamine cycle is a major energy source for tumor cells, including brain tumors (Marin-Valencia et al., 2012; Fendt et al., 2013; Herranz et al., 2015; Tardito et al., 2015). Therefore, targeting the metabolism in GSCs and tumor-associated cells in the TME has recently become one of the most exciting and promising fields for the development of new anticancer treatments (Diwakar et al., 2017).

Selenium Deficiency in Malignant Brain Tumors

Selenium (Se) is a well-known essential trace element that takes part in many physiological processes, such as aging, immune system function, and anti-oxidant defense with the ability to promote neuronal cell survival (Brauer and Savaskan, 2004; Ray et al., 2006; Reid et al., 2008; Naziroglu et al., 2014; Yakubov et al., 2014; Cardoso et al., 2015). Several studies reported significant low Se levels in patients with high-grade gliomas (HGGs, WHO grades III and IV) (Philipov and Tzatchev, 1988; Al-Rawi et al., 2018; Stojsavljevic et al., 2020). A conventional treatment may even aggravate Se deficiency in patients with HGGs (Zeng et al., 2012) and, consequently, this may promote a negative impact on oxidative stress, immune function, and treatment resistance (Yakubov et al., 2014).

In the past century, it has been observed that reversing Se deficiency in patients with brain tumors improves the condition of patients with neurological side effects such as nausea, unsteady gait, speech disorders, or seizures (Philipov and Tzatchev, 1990; Pakdaman, 1998). These patients were given either 1,000 μg inorganic Se in form of sodium selenite by infusion in physiological saline per day for 4–8 weeks (Pakdaman, 1998), or 150 μg organic Se in combination with 60 IU vitamin E for several weeks to 1 year (Philipov and Tzatchev, 1990). This was also demonstrated in rodent xenograft-glioma model (Hervouet et al., 2013; Yakubov, 2019). Se-excessive diet and intrathecal treatment of Se were associated with a prolonged survival and delayed neurological deficits compared to controls or dietary Se-deficient animals (Yakubov, 2019). Similarly, Hervouet et al. (2013) reported a beneficial neurological effect of a diet mixture of α-tocopherol, β-carotene, Se, vitamin C, and zink. Remarkably, the invasive morphology of malignant cells and the tumor aggressiveness were decreased after treatment of Se in both xenograft models (Hervouet et al., 2013; Yakubov, 2019).

Selenium Effects in Glioblastoma Cells

The anti-gliomagenic and neuroprotective effect of Se appeared to have a role in regulation of calcium channels, in particular, transient receptor potential (TRP) cationic channels (**Figure 2**), including TRP melastin 2 (TRPM2) and vanilloid 1 (TRPV1) (Ataizi et al., 2019; Ertilav et al., 2019; Naziroglu et al., 2020; Akyuva et al., 2021). The involvement of TRPM2 channel

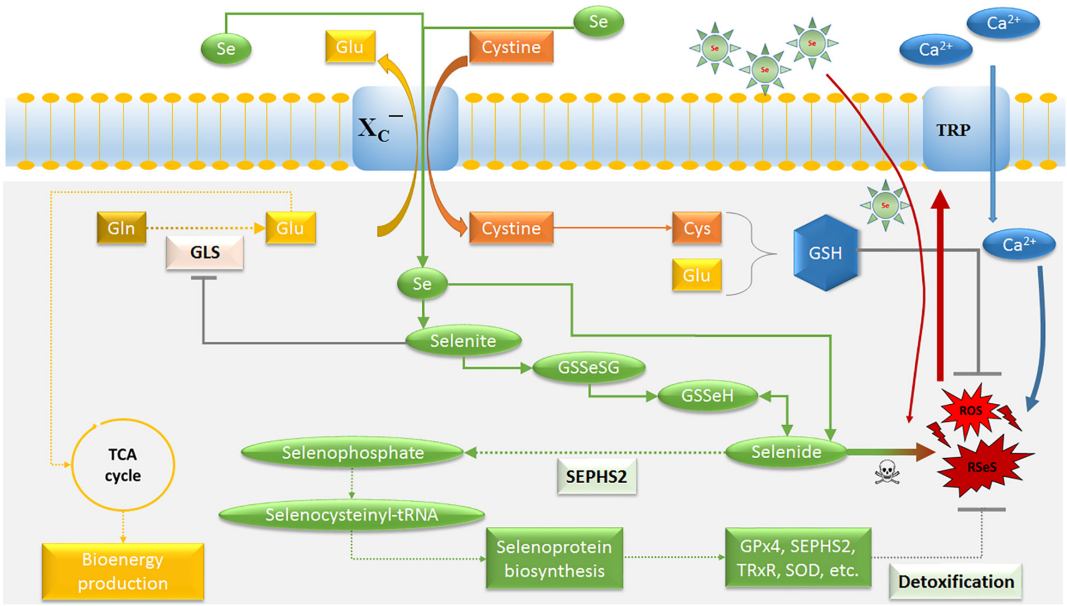

FIGURE 2 | Schematic model for the potential of selenium on glioblastoma cells. The main purpose of the system X_C^- transporter is to supply cysteine for the production of the cellular antioxidant GSH. Selenide forms RSeS. Excessive Ca^{2+} influx through Se-induced oxidative stress causes an activation of TRP channels and mitochondrial membrane depolarization, leading to excessive ROS production. Thus, SEPHS2 is essential for survival in glioblastoma cells due to their elevated expression of system X_C^- transporter, which induces the import of Se compounds selenite and its conversion to toxic selenide resulting in selenide poisoning and cancer death. The increasing import of Se can be achieved by Se-containing nanoparticles. GLS is a key enzyme for glutaminolysis and bioenergy metabolism, which can be inhibited by selenite. Ca^{2+}, calcium; Cys, cysteine; GLS, glutaminase; Glu, glutamate; Gln, glutamine; GSH, glutathione; GSSeSG, selenodiglutathione; GSSeH, selenoglutathione; SEPHS2, selenophosphate synthetase 2; ROS, reactive oxygen species; RSeS, reactive selenium species; Se, selenium compounds; TCA, tricarboxylic acid cycle; GPx4, glutathione peroxidase 4; TRP, transient receptor potential cationic channel; TRxR, thioredoxin reductase; SOD, superoxide dismutase.

on glioblastoma progression was recently reported (Alptekin et al., 2015; Ertilav et al., 2019). Interestingly, Naziroglu's group reported that Se-species stimulate glioblastoma cell death by increasing the mitochondrial ROS generation and the intracellular free calcium concentration through enhanced TRPM2 activation (Ertilav et al., 2019). Nonetheless, protective effects were observed in non-malignant cells such as neurons or microglia cells (Ataizi et al., 2019; Ertilav et al., 2019; Akyuva et al., 2021), suggesting a cellular specificity and higher affinity of Se-species on apoptosis and oxidative stress to glioma cells (Yakubov et al., 2014; Hazane-Puch et al., 2016; Harmanci et al., 2017; Yakubov, 2019). It has been previously reported that low doses of organic and inorganic forms of Se-species have antioxidant properties in malignant cell lines, but there are apoptotic effects with high dose applications (Uguz et al., 2009; Harmanci et al., 2017).

According to Carlisle et al. (2020), a selenium-specific impact on drug-resistant cells selectivity is connected with xCT and selenophosphate synthetase 2 (SEPHS2). SEPHS2 (also known as SPS2) is required for the production of selenoproteins (**Figure 2**), a group of at least 25 proteins containing selnocysteine residues (Xu et al., 2007), which include antioxidant enzymes such as glutathione peroxidase, thioredoxin reductase, and superoxide dismutase (Papp et al., 2007; Brigelius-Flohe, 2008; Yakubov et al., 2014). Glutathione peroxidase 4 (GPx4) has been implicated in the protection of cancer cells against ferroptosis and chemotherapeutic resistance (Yang et al., 2014; Hangauer et al., 2017). However, GPx4 is also required for the detoxification of Se, in particular of the Se-xCT-SEPHS2 pathway intermediate selenide (Carlisle et al., 2020). Inhalation of hydrogen selenide is reported to be toxic (Schecter et al., 1980). Also, it has been suggested that selenide reacts with water during its decay into elemental Se, forming reactive Se and oxygen species (RSeS/ROS) such as hydroxy radicals, superoxide and hydrogen peroxide (Seko and Imura, 1997; Peyroche et al., 2012), suggesting a potential mechanism for its toxicity (**Figure 2**). This was confirmed by a report which showed an improved pharmacologically potential of synthetic selenocyanates in glioblastoma cells as compared to the naturally occurring phenylalkyl isothiocyanates (Sharma et al., 2008). Increasing lipophilicity or isosterically replacing sulfur with Se in the structure-activity of the original precursor compounds, enhanced the toxicity of Se toward glioma cells by affecting their cell redox state. Thus, SEPHS2 is essential for survival in GSCs because of its elevated expression of xCT. The overexpression of xCT leads to the import of dietary Se compound selenite and converts it into toxic selenide, resulting in selenide poisoning and cancer death (Carlisle et al., 2020). Due to the fact that normal cells do not considerably express xCT, and do not depend on SEPHS2 detoxification, they remain surprisingly spared. Moreover, the ability of Se to counteract glutamate release is connected with the inhibition of redox-sensitive transcription

factors, mainly the nuclear factor kappa B (NF-κB) and hypoxia-inducible factor 1 (HIF-1) (Savaskan et al., 2003), reducing glutamate- and hypoxia-induced ROS production in TZ II and TZ III (Mehta et al., 2012). These aspects confirm the existence of a redox reprogramming in GSCs, which differs from, but is possibly influenced by, the other cellular components in the TME.

Selenium-Containing Nanotreatment of Glioblastoma

The beneficial and anti-gliomagenic effects of Se depends on its dose and routes of administration (Weekley and Harris, 2013; Yakubov et al., 2014; Rayman, 2020). Having this in mind, the application of nanotechnology enhanced the therapeutic efficiency of Se-species and reduced side effects on normal cells (He et al., 2017; Ferreira et al., 2019; Geoffrion et al., 2020). Nanoparticle delivery systems with Se-carrier were designed to overcome the blood-brain barrier for the selective treatment of HGGs (You et al., 2016; Song et al., 2018). Recently, Jiang et al. (2014) developed polysaccharide from *Gracilaria lemaneiformis* conjugated to Se nanoparticles. The anti-gliomagenic effects in U87 and C6 glioma cell lines were significantly enhanced by recognizing the $\alpha_v\beta_3$ integrin receptor. There is a higher expression of the integrin receptor in U87 cells than C6 cells, which leads to achieve a higher uptake of inorganic Se by U87 cells via receptor-mediated endocytosis, subsequently inducing and enhancing the production of ROS (**Figure 2**). This leads to glioblastoma cell apoptosis by activating p53 and MAPK cell signal pathways (Jiang et al., 2014). Other laboratory studies confirmed similar results in glioblastoma treatment with some modifications in surface decorating ligands for Se nanoparticles like HER2 and arginylglycylaspartic acid (You et al., 2016; Jardim et al., 2017; Song et al., 2018). Also, it has been demonstrated that Se-containing nanoparticles treatment significantly reduced both the bioenergy metabolism and the invasion of drug-resistant glioma cells (Xu B. et al., 2020), while benign cells remained viable indicating that Se toxicity is selective for glioma cells.

In order to further enhance the therapeutic effect, additional studies have extended the advantages of Se-containing nanomechanisms to deliver chemotherapeutic drugs at a higher concentration. Cheng et al. (2012) demonstrated a superior anti-tumor activity by incorporating Se into temozolomide (TMZ). TMZ-resistant tumor cells could be also effectively be treated with this compound. By comparing TMZ and TMZ-Se, the researchers demonstrated the properties of TMZ-Se as a compound that is able to trigger cell-death more rapidly, with a high apoptosis-inducing activity and as a compound that induces a stronger autophagic response.

Selenium and Heat Shock Protein in Glioma

Heat shock proteins (HSPs) are highly conserved, ATP-dependent chaperone molecules which are expressed rapidly under stress conditions and form a protective microenvironment necessary for gliomagenesis (See et al., 2011; Jego et al., 2013; Iglesia et al., 2019). Glioma microenvironment condition and notable Se deficiency significantly promote the expression of many HSPs – particularly HSP70 and HSP90 – leading to drug resistance (Alexiou et al., 2014; Beaman et al., 2014; Wu et al., 2016; Zhang et al., 2020a,b). In this respect, most of the studies showed that HSPs are actively involved in glioma-related angiogenesis, energy metabolism, and aggressive glioma by the activation of survival pathway such as PI3K/Akt signaling pathway (Choi et al., 2014; Rajesh et al., 2017, 2019). Interestingly, the treatment of glioblastoma cells by antioxidant Se has been shown to decrease oxidative stress and, as a result, HSP expression could be decreased as well (Zhang et al., 2020a).

Previous reports demonstrated that sodium selenite downregulates histone deacetylase (HDAC) activity in glioblastoma cells in a dose-dependent manner (Hazane-Puch et al., 2016). In consequence, this leads to a caspase-3-dependent apoptosis, cell cycle arrest at the G2 phase, and decreased MMP2 activities. The downstream targets of HDACs, HSP90, is also downregulated in malignant cells. The inhibition of tumor HSPs by Se supplementation results in degradation of oncoproteins such as Akt, RAF-1, and VEGFR (Chan et al., 2006; Fu et al., 2016; Hazane-Puch et al., 2016; Yakubov, 2019). Notably, Se-containing derivates of synthetic HDAC inhibitor SAHA (suberoylanilide hydroxamic acid) were significantly more effective in inducing cytotoxicity in different cancer cells than SAHA alone (Desai et al., 2010b; Karelia et al., 2010; Gowda et al., 2012).

Heat shock proteins have attained a great significance in glioma immunotherapy due to their ability to regulate the M2-like polarization of glioma-associated microglia/macrophages (Zhang et al., 2016). Also, an increased immunogenicity of tumor-associated antigens stimulates both innate and adaptive immunity (Moseley, 2000; Gastpar et al., 2005; Specht et al., 2015). Recent randomized clinical trials of vaccination with autologous tumor-derived HSP-peptide complex have been shown to improve survival in patients with newly diagnosed and recurrent glioblastoma (Crane et al., 2013; Bloch et al., 2014; Ji et al., 2018). Interestingly, vaccine nanoformulations allowed combining Se nanoparticles with siRNA and HSP70, increasing their anticancer activity and selectivity between malignant and healthy cells (Li et al., 2016). The development of innovative administration routes and the advances in creating more efficient and safe carriers with Se-containing derivates opened new doors to treatment possibilities against brain malignancies that needed to be further explored and researched.

SELENIUM AND ANGIOGENESIS

The influence of TME on glioblastoma cell behavior plays a crucial role leading to diffuse tumor growth and its invasive capacity. The presence of low tumor oxygenation, also known as hypoxia, strongly correlates with glioma invasiveness (Evans et al., 2004). Hypoxia is also a well-known stimulus for angiogenesis (Carmeliet and Jain, 2000; Seidel et al., 2010). The hypoxic niche is enriched by GSCs due to glioma-induced vascular abnormalities and it induces resistance to drugs and radiation in HGGs (Alexandru-Abrams et al., 2014). A fundamental cellular process, which

occurs subsequent to hypoxia, is the activation of HIF-1 (Guillemin and Krasnow, 1997).

Importantly, it has been recently shown that Se reduced tumor-related angiogenesis by inhibition of angiogenic factors via the suppression of the PI3K/Akt/HIF-1 signaling pathway (Liu et al., 2016; Yakubov, 2019). It must be emphasized that sodium selenite, even at high concentrations (50 μM), had no influence on the angiogenesis of the healthy brain parenchyma and even had a cytoprotective effect in *ex vivo* glioma-induced brain slices. Furthermore, this inorganic Se compound inhibited the growth of malignant gliomas and reduced the development of tumor-related vascular formation in TZ II (Yakubov, 2019). It could be demonstrated that migration patterns of glioblastoma cells along the peritumorally formed tumor-induced microvascularization was significantly inhibited by selenite in a concentration-dependent manner. There is also evidence that selenite can decrease epidermal growth factor receptor (EGFR) expression, leading to apoptosis and comprehensive alterations in the expression of matrix metalloproteinases (MMPs) (Rooprai et al., 2007). Although most MMPs (except MMP-25) were decreased, it could be seen that their natural inhibitor, tissue inhibitor of metalloproteinase (TIMP), increased. The findings of Yoon et al. (2001) reported as well that Se can contribute to prevent migration of endothelial cells through the extracellular matrix (ECM) and inhibit MMP expression and tumor invasion.

Besides, a growing body of evidence shows that glutamate is able to regulate arteriole diameter, blood-brain barrier disruption and vasodilation (Sharp et al., 2003; Liu et al., 2010; LeMaistre et al., 2012; Fan et al., 2017; Peyton et al., 2018). The dependency of glioblastoma and tumor-associated endothelial cells on glycolysis, but also glutaminolysis for energy production, opens further opportunities to reduce tumor-related angiogenesis (Seyfried et al., 2015; Artzi et al., 2017). Glutamine consumption is often increased in malignant tumors and the inhibition of intracellular glutaminase (GLS) activity – which converts glutamine into glutamate – has been shown to reduce proliferation and angiogenesis of tumor cells of different origin (Draoui et al., 2017; Zhao et al., 2017; Bruntz et al., 2019; Restall et al., 2020). Recent studies have demonstrated that Se-induced inhibition of glutaminolysis in malignant cells resulted from the suppression of GLS activity (Zhao et al., 2017; Bruntz et al., 2019). These findings suggest that selenite inhibits GLS activity leading to a decreased bioenergy metabolism and GSH synthesis in cancer and tumor-associated endothelial cells (**Figure 2**). As a consequence, a low level of intracellular glutamate prevents endothelial cell proliferation, resulting in impaired tumor-related angiogenesis (Kim et al., 2017), and accelerating selenide-dependent cancer death (Carlisle et al., 2020).

SELENIUM AND GLIOMA-ASSOCIATED MICROGLIA

Microglia are macrophage-like cells that are considered the major immune cells in the brain (Prinz et al., 2017). Most of the immune cells in HGGs consists of microglia/macrophages, which can sometimes equal the number of tumor cells (Morantz et al., 1979; Roggendorf et al., 1996). Also, it has been suggested that neoplastic microglia/macrophages with phagocytic properties, arising through possible fusion hybridization, can comprise an invasive cell subpopulation within glioblastoma (Huysentruyt et al., 2011). Nevertheless, the role of microglia in tumor progression has been shown to be double-edged, as these cells can both promote tumor rejection and stimulate tumor growth depending on their current functional phenotype (Lisi et al., 2014). Two phenotypes have been described as classically activated microglia (M1), considered a pro-inflammatory, and alternatively activated microglia (M2) a pro-angiogenic, immunosuppressive (Ding et al., 2014). However, the validity of this distinction has been debated (Ransohoff, 2016).

Glioma-associated microglia/macrophages (GAMs) are a major component of tumor infiltrates resulting from either resident microglia or monocytes-derived macrophages (MDMs) from the blood (Li and Graeber, 2012; Glass and Synowitz, 2014; Cai et al., 2020). The tumor-supportive M2-polarization of GAMs seemed to predominate in TZ I and TZ II (Ellert-Miklaszewska et al., 2013; Choi et al., 2015; Ghoochani et al., 2016a,b), although anti-tumoral effects of M1-GAMs were also described (Galarneau et al., 2007). However, hypoxia and glutamate-induced excitotoxicity have been found to play an essential regulatory role in immune response modulation (Laoui et al., 2014; Choi et al., 2015; Henze and Mazzone, 2016). The activation of transcription factors, mainly HIF-1 and NF-κB, are pivotal molecular pathways involved in tumor cells (Mantovani et al., 2008; Yakubov, 2019). These transcription factors modulate the production of macrophage migration inhibitory factor (MIF) and cyclooxygenase-2 (COX-2), leading to overexpression of prostaglandin E2 (PGE2). Immune cells, especially microglia/macrophages are recruited and activated through these transcription factors (Ghoochani et al., 2016a), which leads to glutamate and cytokine release, ROS generation, and overload of calcium influx (Dalla Puppa et al., 2007; Socodato et al., 2018; Yildizhan and Naziroglu, 2020). However, NF-κB is highly dysregulated in HGGs and GAMs (Garkavtsev et al., 2004; Nam et al., 2008; Zanotto-Filho et al., 2017).

It has been shown in brain malignancies that microglia/macrophages can lead to a production of angiogenic factors such as VEGF. This is important for tumor progression through different signaling pathways such as HIF-1/ROS and NF-κB (Brandenburg et al., 2016; Schuett et al., 2017; Blank et al., 2020). Interestingly, studies have demonstrated that Se inhibits the activation of NF-κB in microglia/macrophages (Savaskan et al., 2003; Nam et al., 2008; Xu J. et al., 2020). It is plausible that a disturbance of NF-κB in microglia/macrophages by Se supplementation can lead to a decrease of the local immune suppression and may also affect the expression of cell survival factors such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), MIF, COX-2, and PGE2 (Xin et al., 2010; Fujita et al., 2011; Li et al., 2018; Liang et al., 2018; Cheng et al., 2020). Recently, it has been shown that Se is able to suppress the proinflammatory activity, and mitochondrial stress via inhibition of calcium channels such as TRPM2 channel (Akyuva et al., 2021). Additional supplements of selenoproteins were also associated with an increasement of migration and phagocytosis

properties of microglial cells (Meng et al., 2019). However, COX-2 appears to be one of the key factors regulated by Se (Desai et al., 2010a). There is evidence that Se-dependent inhibition of COX-2 activity with subsequent PGE2 reduction can affect possible cell fusion hybridization in malignant cells (Filippova and Nabors, 2020), resulting in inhibition of glioblastoma growth *in vitro* and *in vivo* (Joki et al., 2000; Fujita et al., 2011; Altinoz et al., 2018). Moreover, GAMs produce high levels of PGE2 through the expression of COX-2 (Badie et al., 2003). Recently, it has been shown that Se through selenoprotein expression decreases the production of PGE2 in microglia/macrophages (Vunta et al., 2008). PGE2 was shown to affect the expression of programmed cell death ligand 1 (PD-L1). This particular expression is associated with tumor progression in gliomas (Litak et al., 2019).

Although these researches pointed Se as a relevant micronutrient in the treatment of brain malignancies, further investigations on the efficacy of Se TME and glioma-associated microglia cells are required.

CONCLUSION

Despite the recent technical improvements in neuro-oncology and oncologic neurosurgery, glioblastoma still remains a lethal medical condition. Se has been independently reported as a promising trace element with anticancer properties. This review provides a consolidated overview of Se potential in glioma microenvironment. On the molecular level, there are evidences that Se operates directly on the redox homeostasis and via selenoprotein regulation. Through its intriguing biology, this trace element holds a center stage in glioblastoma. Se affects bioenergy metabolism, modulates the immunological response, and inhibits tumor angiogenesis. Considering the researches in relation to the potential of Se, it can be concluded that Se represents a promising agent in neuro-oncology.

However, the insights of this review arise further questions in relation to the paradoxical effects of this micronutrient. Challenging for further researchers addressing the detrimental effects of Se could be the fact that Se deficiency in patients with malignant gliomas are more common. Also, it would be worthwhile a further mechanistic glimpse into the role of Se compounds in TME and tumor-associated cells such as GAMs. The creation of innovative Se derivates in nanomedicine approaches provide new therapeutic weapons against glioblastoma and expand the range for new researches. Novel nanomedicine Se-derivates can overcome the low therapeutic range and selectivity of Se and improves the general cytotoxic profile in normal cells.

AUTHOR CONTRIBUTIONS

EY designed the structure and contents of the review and wrote the manuscript. EY and TE prepared all figures. All authors provided critical revisions to the article and contributed to the article and agreed to submit the manuscript in its current state.

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ORIGINAL RESEARCH
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Selenium-Binding Protein 1 (SELENBP1) as Biomarker for Adverse Clinical Outcome After Traumatic Spinal Cord Injury

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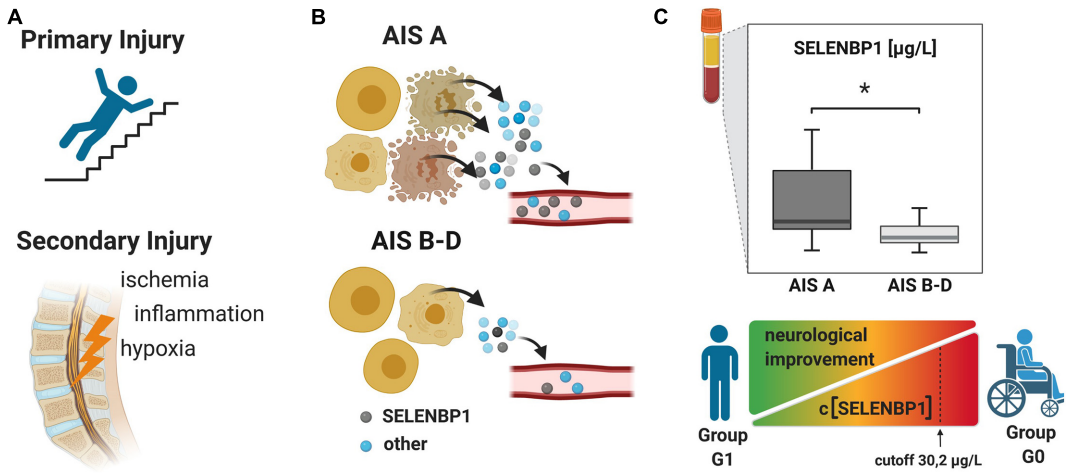
Introduction: Traumatic spinal cord injury (TSCI) presents a diagnostic challenge as it may have dramatic consequences for the affected patient. Additional biomarkers are needed for improved care and personalized therapy.

Objective: Serum selenium binding protein 1 (SELENBP1) has been detected in myocardial infarction, reflecting hypoxic tissue damage and recovery odds. As SELENBP1 is usually not detected in the serum of healthy subjects, we tested the hypothesis that it may become detectable in TSCI and indicate tissue damage and regeneration odds.

Methods: In this prospective observational study, patients with comparable injuries were allocated to three groups; vertebral body fractures without neurological impairment (control "C"), TSCI without remission ("G0"), and TSCI with signs of remission ("G1"). Consecutive serum samples were available from different time points and analyzed for SELENBP1 by sandwich immunoassay, for trace elements by X-ray fluorescence and for cytokines by multiplex immunoassays.

Results: Serum SELENBP1 was elevated at admission in relation to the degree of neurological impairment [graded as A, B, C, or D according to the American Spinal Injury Association (AISA) impairment scale (AIS)]. Patients with the most severe neurological impairment (classified as AIS A) exhibited the highest SELENBP1 concentrations (p = 0.011). During the first 3 days, SELENBP1 levels differed between G0 and G1 (p = 0.019), and dynamics of SELENBP1 correlated to monocyte chemoattractant protein 1, chemokine ligand 3 and zinc concentrations.



GRAPHICAL ABSTRACT | The pathophysiology of traumatic spinal cord injury (TSCI) can be divided into two major phases. **(A)** The mechanical trauma is followed within minutes by a secondary phase consisting of local complex and intertwined acute responses, intercellular signaling and cell activity regulating pathways. Inflammatory processes, oxidative stress and hypoxia, leading to cell damage and death, and specific cell contents are released into the circulation **(B)**. The motor and sensory deficits upon TSCI are assessed by using the American Spinal Injury Association (ASIA) impairment scale (AIS), ranging from AIS A as a complete absence of any motor and sensory functions under the lesion site, to AIS E with complete preservation of motor and sensory functions. **(C)** The concentrations of serum SELENBP1 were elevated in patients classified as AIS A as compared to less severely affected patients classified as AIS B, C or D. A cut-off was deduced [(SELENBP1) > 30.2 µg/L], reliably predicting whether a patient belongs to the group showing neurological recovery (G1) or not (G0) within 3 months after the trauma. The figure was created by using https://biorender.com.

Conclusion: Circulating SELENBP1 concentrations are related to the degree of neurological impairment in TSCI and provide remission odds information. The tight correlation of SELENBP1 with CCL2 levels provides a novel link between Se metabolism and immune cell activation, with potential relevance for neurological damage and regeneration processes, respectively.

Keywords: diagnostic biomarker, *in vitro* diagnostic test, trace element, neuroregeneration, neurotrauma

INTRODUCTION

Traumatic spinal cord injury (TSCI) remains one of the most severe injuries and affects predominantly young patients (Furlan et al., 2013; Spinal Cord Injury [SCI], 2016). On a pathophysiological level, the primary injury phase is characterized mainly by the mechanical disruption of the spinal cord (SC) due to shearing, laceration, acute stretching, and sudden acceleration-deceleration events (Baptiste and Fehlings, 2006; Rowland et al., 2008). Hereafter, a secondary injury phase is driven by complex inflammatory responses, involving excitotoxicity, ischemia/hypoxia, inflammation, increased spinal cord intraparenchymal pressure, and oxidative stress. Ultimately, these processes determine the extent of neuronal loss after the mechanical insult (Kwon et al., 2004; Shadgan et al., 2019). Finally, the chronic phase is characterized by adaptive processes, recovery, or autonomic dysregulations (Kwon et al., 2004; Norenberg et al., 2004; Rowland et al., 2008; Moghaddam et al., 2015). Due to the highly dynamic nature of these processes, an informative assessment of the remaining or regained neurological functions after TSCI can only be conducted after months, when a new balance is established. Objective and early biomarkers for the extent of damage with potential relevance for remission and prognosis are urgently needed (Kwon et al., 2019).

The essential trace elements selenium (Se), copper (Cu), and zinc (Zn) are of crucial relevance for immune responses and neurological repair processes (Levenson, 2005; Ma et al., 2018; Zhang et al., 2020) partly due to trace element containing proteins with enzymatic or transport functions. Selenoprotein P (SELENOP) is a circulating Se transport protein with peroxidase activity, ceruloplasmin (CP) is an oxidoreductase and Cu transporter, and the intracellular Cu/Zn superoxide dismutase is an essential component of the antioxidative defense (Besold et al., 2016; Kielczykowska et al., 2018; Lewandowski et al., 2019). Thus, trace elements, when available to the organism in physiological concentrations, exert a beneficial influence on the regulation of various immune cells (Avery and Hoffmann, 2018), are facilitating in regeneration processes after injuries (Lansdown et al., 2007), or, in the form of SELENOP, influence the survival of neurons exposed to oxidative stress (Yan and Barrett, 1998).

Se-binding protein 1 (SELENBP1) is a poorly characterized parameter of Se metabolism, transport and intracellular accumulation. It can exert enzymatic activity, capable of oxidizing methanethiol (Pol et al., 2018), and it constitutes

a potential early biomarker of schizophrenia (Mohammadi et al., 2018). SELENBP1 is located intracellularly under normal conditions, partly in complex with Se-dependent glutathione peroxidase 1 (GPX1) (Diamond, 2015). Its expression is dysregulated in malignant tissue (Hughes et al., 2018; Schott et al., 2018), and it may serve as a biomarker of adipocyte differentiation (Steinbrenner et al., 2019). There are indications that the protein contributes to redox control, affecting cell differentiation and motility (Elhodaky and Diamond, 2018). Moreover, extracellular SELENBP1 can be detected in blood following myocardial infarction or during cardiac surgery, where serum SELENBP1 levels correlate to tissue damage and hypoxic stress (Kühn et al., 2019; Kuhn-Heid et al., 2019).

Based on these findings, we hypothesized that TSCI might be associated with an increase in circulating SELENBP1 concentrations and that elevated serum SELENBP1 at an early stage after injury may correlate to the severity and the neurological outcome of this devastating condition. Accordingly, the aim of this study was to determine circulating SELENBP1 concentrations and to analyze whether this parameter correlates to the extent of neurological impairment and clinical outcome after 3 months. In order to facilitate the evaluation of the analyses and to identify potential associations with other potentially relevant parameters, circulating chemokines, trace elements and associated biomarkers were analyzed in parallel.

MATERIALS AND METHODS

Study Design

This clinical prospective observational study has been approved by the local ethics committee of the University of Heidelberg (S514/2011). It was registered (Study-ID: DRKS00009917/ Date of Registration: 23.03.2016/Universal Trial Number (UTN): U1111-1179-1620) at the German Clinical Trial Register (Deutsches Register Klinischer Studien—DRKS). Data collection and processing were performed according to good scientific practice, and the manuscript was composed according to the STROBE statement (von Elm et al., 2008). All study participants signed an informed consent form and agreed to participate. The patients were informed that they could choose to leave the study without reason at any time and that this decision will not affect further treatment in any way.

Source of Clinical Data

The clinical data were collected during the examinations and consecutively provided by the hospital database. Inclusion criteria were defined as the occurrence of at least one fracture of the spine with accompanying sensorimotor deficits resulting from TSCI. Fractures were classified according to the AO classification (Magerl et al., 1994) and the occurrence of sensorimotor deficits described as neurological level of injury (NLI). The NLI is defined as the lowest neurological level, where both motor and sensory functions are intact. Exclusion criteria were non-traumatic spinal cord injury (SCI), traumatic brain injury, severe abdominal trauma, traumatic amputation of extremities, coma, or any additional life-threatening trauma apart from the SCI (Heller et al., 2020). During the study period, no methylprednisolone sodium succinate was provided to the participating patients. The patients included in the study were grouped into the study group S (n = 34), which was retrospectively divided into two subgroups G0 and G1 according to the clinical outcome after 3 months. G1 (n = 19) included patients with neurological remission, and group G0 (n = 15) consisted of patients without any improvement of the neurological functions within 3 months after injury. Ten subjects with vertebral fractures without neurological impairment were analyzed and served as the control group C (n = 10). The detailed patient allocation to the groups is visualized (**Figure 1**), and patient characteristics are provided (**Tables 1**, **2**).

Source of Material

Venous blood samples were collected in the Department of Paraplegiology at the BG Trauma Center Ludwigshafen from TSCI patients from 2011 to 2018. Consecutive blood samples were drawn from patients at specific time points covering the period from the time of admission until 3 months after injury according to our study protocol (**Figure 2**). All blood samples were treated routinely according to the same standard procedure; 20 min of coagulation at room temperature, centrifugation at 3,000 rpm with an RCF of 1,000 g, aliquoting into sterile tubes and storing at −80°C until analysis or transport on dry ice. Missing samples in the protocol are mostly due to urgent interventions. The laboratory analyses for SELENBP1 and cytokine concentrations were conducted by staff blinded to patient identities and clinical data in the Institute for Experimental Endocrinology of the Charité–Universitätsmedizin Berlin and the Institute of Immunology at Heidelberg University Hospital, respectively.

Sample Analysis

Trace Element Analyses

Trace element concentrations were determined by total reflection X-ray fluorescence (TXRF) analysis, essentially as described (Hughes et al., 2016; Heller et al., 2019). Briefly, serum samples were diluted with a Gallium standard and applied to polished quartz glass discs. After drying, a benchtop TXRF device (PicoFox S2, Bruker Nano, Berlin, Germany) was used for recording the fluorescence spectra emitted from the elements upon X-ray excitation. An internal laboratory quality control was included in each measurement run, and all samples were measured in duplicate (Heller et al., 2020). The inter-assay coefficient of variation (CV) was below 10%, as determined with a commercial standard serum (Sero AS, Seronorm, Billingstad, Norway) (Hughes et al., 2016; Heller et al., 2019).

Ceruloplasmin (CP) and Selenoprotein P (SELENOP) Quantification by Sandwich Immunoassays

Serum samples were tested for SELENOP and CP concentrations. To this end, a validated enzyme-linked immunosorbent sandwich assay specific for human SELENOP (selenOtest^{TM}, ELISA) (Hybsier et al., 2017) was used according to the manufacturer's instructions (selenOmed GmbH, Berlin, Germany). Serum CP concentrations were determined by a sandwich ELISA using a

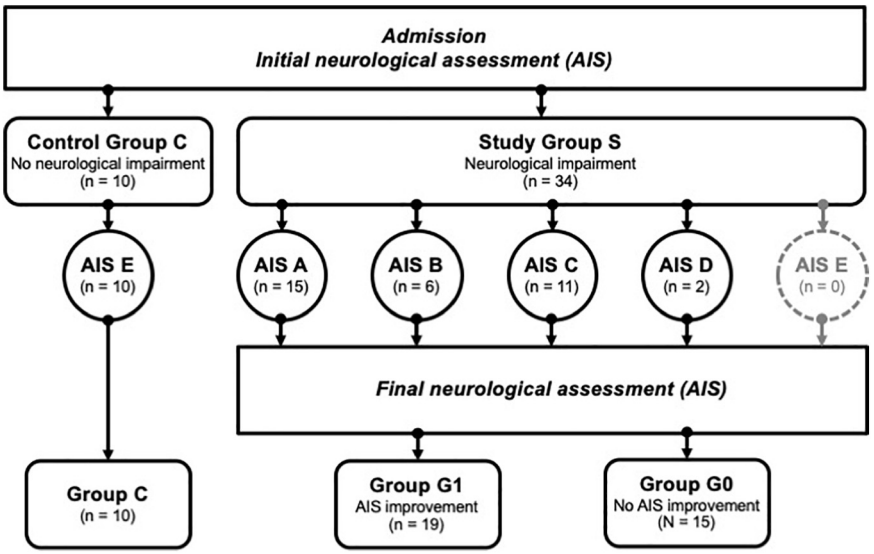


FIGURE 1 | Patient identification and allocation scheme. A set of $n = 44$ patients were successfully enrolled into the study, and divided into groups with no neurological impairment (Control Group C) vs. patients with neurological impairment (Study Group S). The latter group was subdivided further according to the severity of the symptoms using the AIS categories A-D, where A relates to the most severe condition. AIS improvement was assessed 3 months after enrolment, allowing a subdivision of S into group G1 (improvement) or G0 (no improvement). Group C; controls, group G1; patients with neurological remission, group G0; patients without neurological remission, AIS; American Spinal Injury Association (ASIA) Impairment Scale.

pair of specific monoclonal antibodies in combination with a commercial human CP standard (CP, catalog number 187-51, Lee BioSolutions, Maryland Heights, MO 63043, United States) as described earlier (Hackler et al., 2020).

Selenium Binding Protein 1 (SELENBP1) Quantification by LIA

Serum SELENBP1 concentrations were analyzed by a recently established luminometric immunoassay (LIA) (Kühn et al., 2019). Quality of measurements was verified by including two human serum standards in each assay run. Intra- and inter-assay variations of SELENBP1 concentrations were below 15% during the analyses.

Cytokine Quantification via Multiplex Bead-Based Immunoassays

Multiplex bead-based immunoassays were used to quantify a set of human chemokines and cytokines (Luminex Performance Human High-Sensitivity Cytokine Panels). Serum concentrations of CCL-2, CCL-4, MMP-2, MMP-8, IL-8, and IL-10 were assessed. The determination was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, United States).

Outcome

The American Spinal Injury Association (ASIA) impairment scale (AIS) was used to describe the functional impairment in TSCI patients. The neurological functions were graded as A, B, C, or D by experienced examiners applying the International Standards for Neurological Classification of SCI (ISNCSCI). To quantify the neurological deficit according to the AIS various parameters such as sensitivity, motor function, muscle strength, and the level of the paraplegia are considered. Hence, AIS A grade represents the complete loss of all motor and sensory functions below the site of injury. Whereas AIS B-D constitute incomplete deficits with remaining sensory and/or motor qualities, with B containing the least and D the most functions. Physiological findings without neurologic impairment are classified as AIS E (**Table 3**). Initial examinations (AIS initial) were performed within 72 h after admission in awake and responsive patients, and final examinations (AIS final) took place at 3 months after the trauma (Burns and Ditunno, 2001). Neurological remission was defined as an improvement of AIS grades within 3 months after the trauma. The initial AIS is illustrated in **Figure 1** and **Tables 1**, **2**.

Predictors

The individual protein and trace element concentration patterns were analyzed concerning both the initial AIS and the presence or absence of neurological remission within 3 months after the injury (**Table 3**).

Sample Size

Serum samples from this observational study, along with the respective clinical data of the patients have already been analyzed for different parameters in other studies by our research groups. The explorative research studies have been performed with slightly different sets of patient samples, depending on the inclusion criteria combined with the respective availability of a sufficient quantity of serum samples stored in the biobank at the time of analysis. For this reason, the numbers of patients

TABLE 1 | Descriptive depiction of patient characteristics of subjects in the study group S and the control group C.

[A]	Study Group S (*N* = 34)	[B]	Control Group C (*N* = 10)
Age		**Age**	
Median	41.0	Median	41.0
(IQR)	(15.0, 77.0)	(IQR)	(27.0, 71.0)
Sex		**Sex**	
Female	8 (23.5%)	Female	5 (50.0%)
MALE	26 (76.5%)	Male	5 (50.0%)
AIS initial		**Etiology**	
A	15 (44.1%)	Fall	7 (70.0%)
B	6 (17.6%)	Traffic	3 (30.0%)
C	11 (32.4%)		
D	2 (5.9%)	**AO**	
		A	7 (70.0%)
AIS final		B	3 (30.0%)
A	11 (32.4%)		
B	3 (8.8%)		
C	6 (17.6%)		
D	14 (41.2%)		
Etiology			
Fall	20 (58.8%		
Other	3 (8.8%)		
Traffic	11 (32.4%)		
NLI			
Cervical	13 (38.2%)		
Lumbar	8 (23.5%)		
Thoracic	13 (38.2%)		
AO*			
A	20 (60.6%)		
B	5 (15.2%)		
C	8 (24.2%)		

*NLI, Neurological Level of Injury; AO, AO-Classification; AIS, ASIA (American Spinal Injury Association) Impairment Scale. Age is expressed as median years with their corresponding IQR. *One patient in the subgroup G0 suffered an isolated contusion of the spinal cord without vertebral fracture, so no AO classification was assessed.*

and samples vary across the different analyses, according to availability, volumes, and the specific scientific issue.

Missing Data

The mean follow-up of available serum samples for analysis within the first 3 days was higher than 75%; missing values were excluded from the pairwise deletion (Kang, 2013).

Statistical Analysis

Non-parametric test methods were assessed to investigate location shifts between groups (Mann-Whitney *U*-test, Kruskal-Wallis test). Categorical variables were evaluated using Fisher's exact test.

As this is an exploratory *post-hoc* analysis, all *p*-values are to be interpreted descriptively, and no adjustment for multiple testing was adopted. The statistical tests are using an α-level of 0.05, and statistical significance was defined as $p > 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***). For SELENBP1 at admission, an optimal cut-off for the differentiation between G0 and G1 was estimated based on the Odds Ratio (OR). All statistical calculations were performed with R version 4.0.2 (R Development Core Team, 2015). Figures were created by using the package "ggplot2" (Wickham, 2009).

RESULTS

A total of 44 patients were eligible for the current study, including 34 patients with neurological impairment (study group S), divided into 19 patients with remission (group G1) and 15 patients without remission (group G0). The other 10 subjects with vertebral fracture without neurological impairment (group C) served as a control group (**Figure 1**).

Patients

Out of all 34 patients in the study group S (G0+G1), eight were female, and 26 were male with an average age of 41 years (IQR 15, 77 years). The TSCI was caused by a fall in 59% of cases, and by accident in 32% of cases. The injuries in the control group (including five males and five females with an average age of 41 years) resulted from a fall in about 70% of cases or from an accident in the remaining 30% of cases. Within the study group S, there were no significant differences regarding age, sex, etiology, NLI or AO classification between the patients with and without neurological remission. The distribution of AIS grades between the groups G0 and G1 differed significantly both at admission ($p = 0.001$) and at discharge ($p < 0.001$). An overview of the patients' characteristics is shown in **Tables 1**, **2**.

Biochemical Analysis of the Serum Samples

The analysis of the serum samples indicated that Se, Zn, Cu, SELENOP, SELENBP1, CP, CCL-2, CCL-3, MMP-8, MMP-10, IL-8, and IL-10 were detectable in measurable concentrations. Most of the parameters analyzed displayed impairment-dependent concentration differences according to AIS grades A vs. B-D early in the post-injury period and differed in relation to injury when comparing the groups G0 and G1 vs. C (**Figure 3**).

Major Findings

Serum SELENBP1 Concentrations Are Elevated With the Severity of Impairment

Increased SELENBP1 concentrations were detected especially at early time points available for analysis, i.e., directly at admission to hospital (0 h). The patients with severe injury and an AIS classification of A exhibited relatively high concentrations of SELENBP1 as compared to the other patients, suggesting a relation of acute serum SELENBP1 elevations to the severity of neurological impairment (**Figure 4A**).

Dynamic Changes in Circulating SELENBP1 Are Related to Clinical Outcome

The SELENBP1 concentrations during the first 3 days in relation to the clinical outcome as assessed 3 months after injury differed significantly between the two groups of subjects with TSCI.

TABLE 2 | Clinical characteristics of subjects in the groups G0 (n = 15) and G1 (n = 19), and subgroups AIS A (n = 15) and AIS B-D (n = 19).

	G0 (n = 15)	G1 (n = 19)	p-value	AIS A (n = 15)	AIS B-D (n = 19)	p-value
Age			0.107			0.238
Median	47.0	32.0		44.0	34.0	
(IQR)	(21.0, 77.0)	(15.0, 75.0)		(21.0, 75.0)	(15.0, 77.0)	
Sex			1.000			1.000
Female	3 (20.0%)	5 (26.3%)		4 (26.7%)	4 (21.1%)	
Male	12 (80.0%)	14 (73.7%)		11 (73.3%)	15 (78.9%)	
AIS initial			0.001			< 0.001
A	11 (73.3%)	4 (21.1%)		15 (100.0%)	0 (0.0%)	
B	1 (6.7%)	5 (26.3%)		0 (0.0%)	6 (31.6%)	
C	1 (6.7%)	10 (52.6%)		0 (0.0%)	11 (57.9%)	
D	2 (13.3%)	0 (0.0%)		0 (0.0%)	2 (10.5%)	
AIS final			< 0.001			< 0.001
A	11 (73.3%)	0 (0.0%)		11 (73.3%)	0 (0.0%)	
B	1 (6.7%)	2 (10.5%)		2 (13.3%)	1 (5.3%)	
C	1 (6.7%)	5 (26.3%)		2 (13.3%)	4 (21.1%)	
D	2 (13.3%)	12 (63.2%)		0 (0.0%)	14 (73.7%)	
Etiology			0.588			0.588
Fall	9 (60.0%)	11 (57.9%)		9 (60.0%)	11 (57.9%)	
Other	2 (13.3%)	1 (5.3%)		2 (13.3%)	1 (5.3%)	
Traffic	4 (26.7%)	7 (36.8%)		4 (26.7%)	7 (36.8%)	
NLI			0.083			0.083
Cervical	4 (26.7%)	9 (47.4%)		4 (26.7%)	9 (47.4%)	
Lumbar	2 (13.3%)	6 (31.6%)		2 (13.3%)	6 (31.6%)	
Thoracic	9 (60.0%)	4 (21.1%)		9 (60.0%)	4 (21.1%)	
AO*			0.039			0.015
A	5 (35.7%)	15 (78.9%)		6 (40.0%)	14 (77.8%)	
B	4 (28.6%)	1 (5.3%)		5 (33.3%)	0 (0.0%)	
C	5 (35.7%)	3 (15.8%)		4 (26.7%)	4 (22.2%)	

*The Kruskal-Wallis test for comparison of two independent samples and the Fisher's exact test were used to assess the differences in numeric and categorical variables. NLI, Neurological Level of Injury; AO, AO-Classification; AIS, ASIA (American Spinal Injury Association) Impairment Scale. Age is expressed as median years with their corresponding IQR. Neurological remission was defined as improvement in AIS within 3 months after the trauma. *One patient in the subgroup G0 suffered an isolated contusion of the lumbar spinal cord without vertebral fracture, so no AO classification was assessed.*

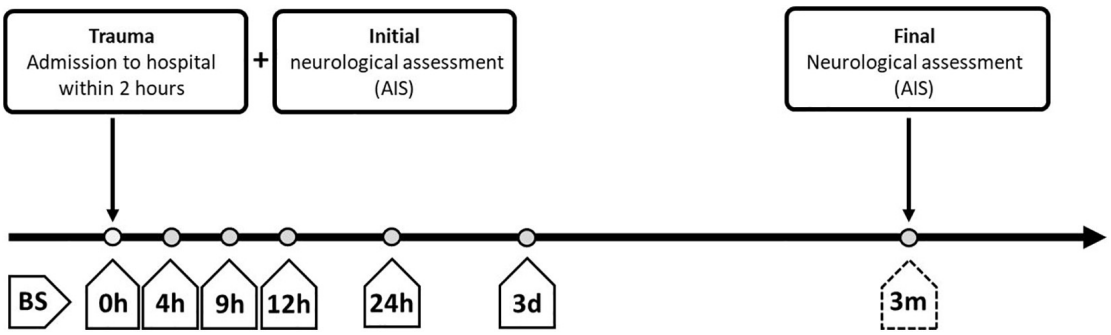


FIGURE 2 | Overview of standardized blood sampling and neurological assessment protocols for patients with severe traumatic injuries. The study enrolled patients who were administered to the hospital within 2 h after the traumatic injury. Blood samples (BS) were collected at different time points after admission, starting immediately at entry into the hospital (0 h after injury; BS 0 h) and extending until the time of final neurological assessment at 3 months after admission (BS 3 m). BS, blood sample; AIS, American Spinal Injury Association (ASIA) Impairment Scale; h, hours; d, days; m, months.

Patients without remission in group G0 showed relatively high SELENBP1 concentrations at admission (0 h), as compared to the patients with remission in group G1, who had low concentrations throughout the full observation period. The elevated SELENBP1 concentrations in G0 decreased steadily within the first 9 h, while the low SELENBP1 levels in G1 remained constant (**Figure 4B**).

A direct comparison of serum SELENBP1 concentrations at the time of admission (0 h) highlights the significantly elevated

TABLE 3 | The American Spinal Injury Association Impairment Scale (AIS).

AIS grade		Clinical state
A	Complete	No motor or sensory function is preserved in the sacral segments S4-S5
B	Incomplete	Sensory but not motor function is preserved below the NLI and includes the sacral segments S4-S5
C	Incomplete	Motor function is preserved below the NLI, and more than half of key muscles below the NLI have a muscle grade less than 3
D	Incomplete	Motor function is preserved below the NLI, and at least half of key muscles below the NLI have a muscle grade of 3 or more
E	Normal	Motor and sensory function is normal

AIS grades from A to E are considering the completeness of paralysis and the motor and sensory function tests.

SELENBP1 levels in relation to the extent of the neurological impairment after TSCI, i.e., the patients with most severe injury classified by the AIS system as A displayed highest SELENBP1 (**Figure 4C**). The Mann-Whitney test indicated that when comparing the initial three samples with the later time points (0 h, 4 h, 9 h vs. 12 h, 24 h, 72 h) the dynamic decrease in SELENBP1 concentrations (Δ SELENBP1) was significantly greater for in G0 (no remission) (Median = 5.32) than G1 (remission) (Median = 0.99), $W = 197$, $p = 0.019$ (**Figure 4D**). Based on the data obtained for serum SELENBP1 concerning remission, a cut-off of 30.2 μg/L was calculated for allocating patients either to G0 or to G1, providing 98.7% sensitivity, specificity of 12.3%, an accuracy of 58.6%, and an odds ratio of 10.4. This diagnostic cut-off is indicated as a solid line, whereas the SELENBP1 level of controls is indicated as a dashed line at 16.2 μg/L (**Figures 4A–C**).

Correlation Analysis of SELENBP1 With Parameters of Se Status and Covariates in TSCI

SELENBP1 concentrations were not significantly related to the other Se status biomarkers. The interrelations were characterized by low correlation coefficients of $R = -0.012$ for SELENBP1 with total serum Se (**Figure 5A**), and $R = 0.110$ for SELENBP1 with SELENOP, respectively (**Figure 5B**). Concerning the outcome of neurological remission, the correlations of SELENBP1 with Se or SELENBP1 with SELENOP tended to point into opposite directions for patients in group G0 vs. G1 (**Figures 5A,B**). These findings suggest that serum SELENBP1 is not a surrogate marker of blood Se status in the patients. As expected, total serum Se and SELENOP showed the typically strong and linear interrelation with a correlation coefficient of $R = 0.76$ in the samples from patients with TSCI, irrespective of final remission (**Figure 5C**). Next, correlations between SELENBP1 concentrations and additional trace elements and cytokines were analyzed to identify other potential covariates. Three parameters correlated significantly with the SELENBP1 decline (Δ SELENBP1; delta 3 day–0 h), i.e., CCl-2 at 0 h ($p = 0.007$, **Figure 5D**), Zn at 9 h ($p = 0.027$, **Figure 5E**) and CCL-3 at 3 day ($p = 0.014$, **Figure 5F**). Concerning the clinical outcome 3 months after TSCI in the group of non-improving patients (G0), strong positive correlations of Δ SELENBP1 were observed for CCL-2 ($R = 0.66$) and CCL-3 ($R = 0.58$), and a negative correlation for Zn ($R = -0.71$). In contrast to these correlations, only moderate and non-significant interrelations were observed in the group of recovering patients in G1 (**Figures 5D–F**).

Relation of Serum SELENBP1 Levels to Trace Elements and Cytokines

An explorative correlation analysis was conducted between the SELENBP1 concentrations with the analyzed trace element parameters and cytokine concentrations. A strong linear association between the initial SELENBP1 concentrations and the dynamics of SELENBP1 decline over the initial 3-day study period was observed (0 h; $R = 0.94$, 4 h; $R = 0.86$, 9 h; $R = 0.58$). To assess differences in all parameters between the groups G0 and G1 with respect to the initial AIS scores, the corresponding log-fold changes (logFC) for each point in time in G1/G0 were calculated. The analysis indicated a negative relationship between SELENBP1 and the group of chemotactic ligands CCL-2, CCL-3, and CCL-4. This interaction was most substantial when comparing the initial concentrations of the most severely impaired patients (AIS group A) in the group with remission (G1) vs. the group with no remission (G0) (**Supplementary Figure 1**).

DISCUSSION

The fundamental need for novel therapies improving the neurological recovery of patients who suffer from SCI remains an urgent research issue. However, specific treatments that appeared promising in a pre-clinical setting regrettably failed to show beneficial effects in clinical SCI trials (Tator, 2006). A reason for the controversial study results may be found in the underlying pathophysiological and biochemical processes that are setting in upon SCI and which may fundamentally differ in extent, dynamics and interrelation between the animal models used and the acutely injured human subjects (Kwon et al., 2015). This challenge is most difficult to address, given the paucity of molecular data on the intracellular signaling events, metabolic responses to trauma in patients and the lack of informative diagnostic biomarkers. In addition, the available instrumentation for estimating the extent of injury and predicting the outcome after a TSCI is limited, and reliability of both diagnosis and prognosis mainly depends on the experience and knowledge of the particular clinical examiner. In order to gain further insights and test candidate biomarkers, we have standardized some of

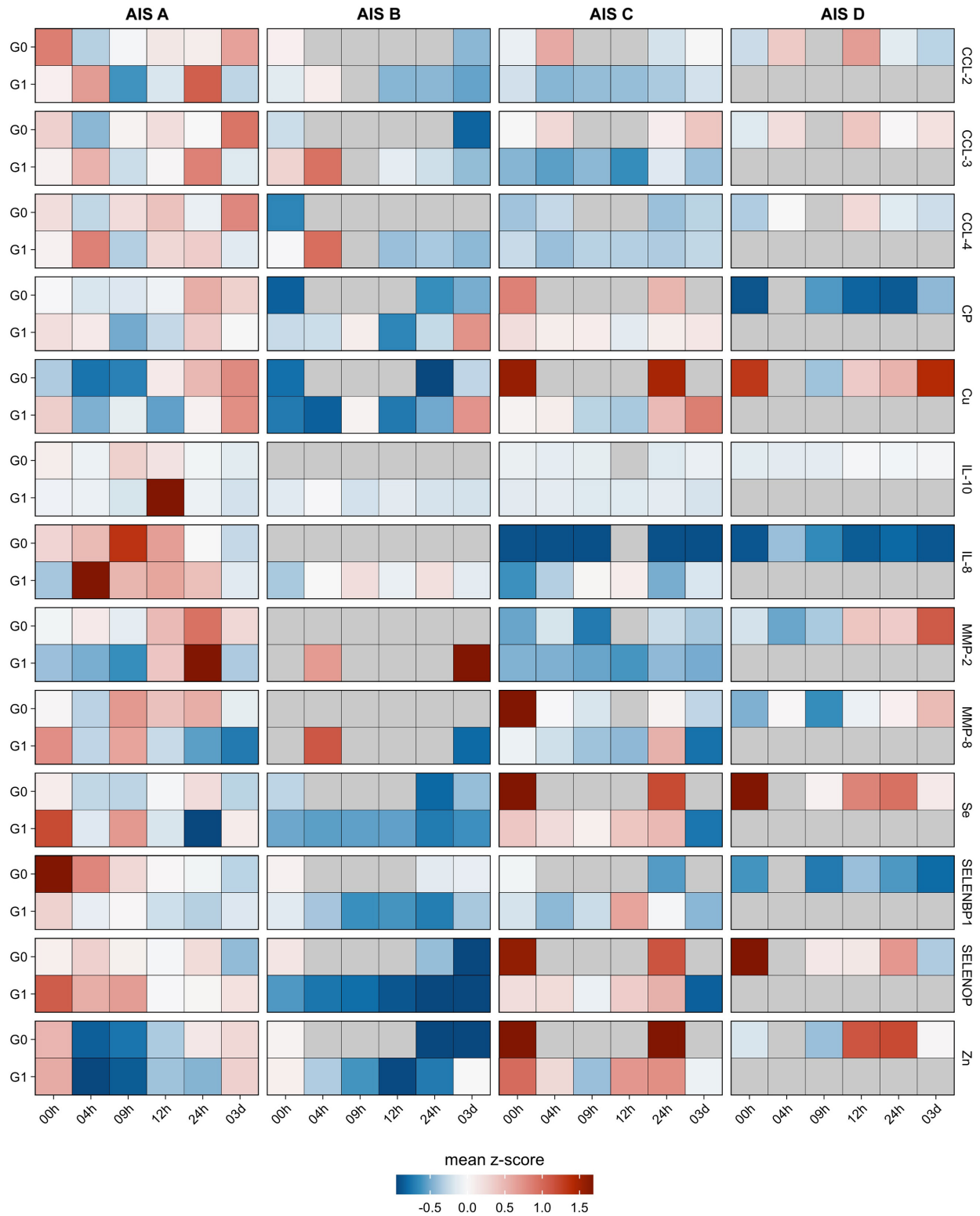


FIGURE 3 | Comparison of cytokines, metalloproteases, trace elements and related biomarkers in relation to neurological remission. The cytokines CCL-2, CCL-3, CCL-4 along with the metalloproteases MMP-2 and MMP-8 were analyzed from the serum samples of patients with TSCI in relation to remission (G1) or no remission (G0). In addition, the trace elements Cu, Fe, Se, and Zn as well as the Se-binding proteins SELENBP1 and SELENOP along with the Cu transporter CP were quantified in parallel. The heat maps indicate the relative concentration differences of these serum parameters in the two groups of TSCI patients (G0 and G1) in relation to the control group C. In addition, relative concentration differences are depicted in regard to the clinical severity of the neurological deficit, classified as AIS A–D according to the American Spinal Injury Association (AISA) impairment scale (AIS). Mean z-scores are indicated as color code.

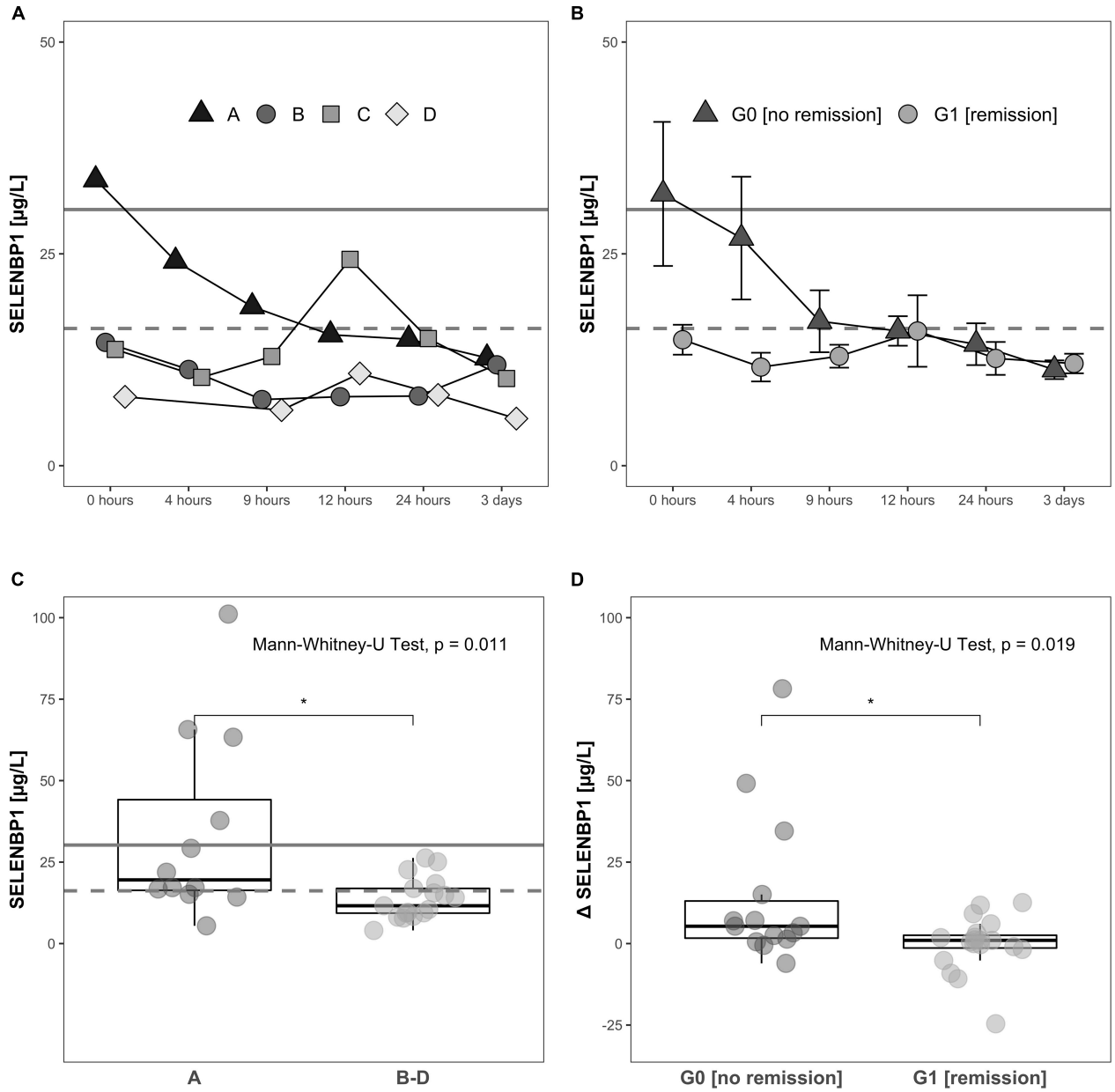


FIGURE 4 | Relation of serum SELENBP1 to the functional impairment at admission and neurological remission. Differences in SELENBP1 concentrations are shown as time-resolved line plots **(A,B)** or box plots **(C,D)**. The mean SELENBP1 levels (16.2 µg/L) of the control group C are depicted as dashed line. Changes (Δ SELENBP1) were calculated by subtracting the late (12 h–3 day) from the early (0–12 h) SELENBP1 concentrations **(D)**. Serum SELENBP1 at hospital admission (0 h) was elevated in patients classified as A by the AIS scheme (most severe) in comparison to patients with a **(B–D)** rating (less severe) **(A,C)**. Similarly, serum SELENBP1 is particularly elevated during the first 3 days after injury in patients not undergoing remission **(B,D)**. The calculated cut-off (30.2 µg/L) for the SELENBP1 threshold as a prognostic marker for remission is indicated as solid line. Values are expressed as mean ± SEM. The Mann-Whitney-U-Test assessed significant differences between two groups; $^*p < 0.05$.

the essential parameters and introduced a transparent blood sampling, clinical assessment and laboratory analysis scheme.

Our results suggest that the analysis of serum SELENBP1 may contribute to an improved initial clinical assessment after TSCI and may provide valuable insights into the pathophysiology and individual prognosis. Significant differences in SELENBP1 were detected with regards to degree of neurological impairment (severe AIS A vs. AIS B-D), and with respect to the clinical outcome after 3 months (remission vs. no remission). The patients who displayed elevated serum SELENBP1 concentrations at admission (above the cut-off at 30.2 µg/L) presented with the most severe impairment (classified as AIS A) and were most unlikely to achieve neurological remission. The other severely impaired patients with the same classification

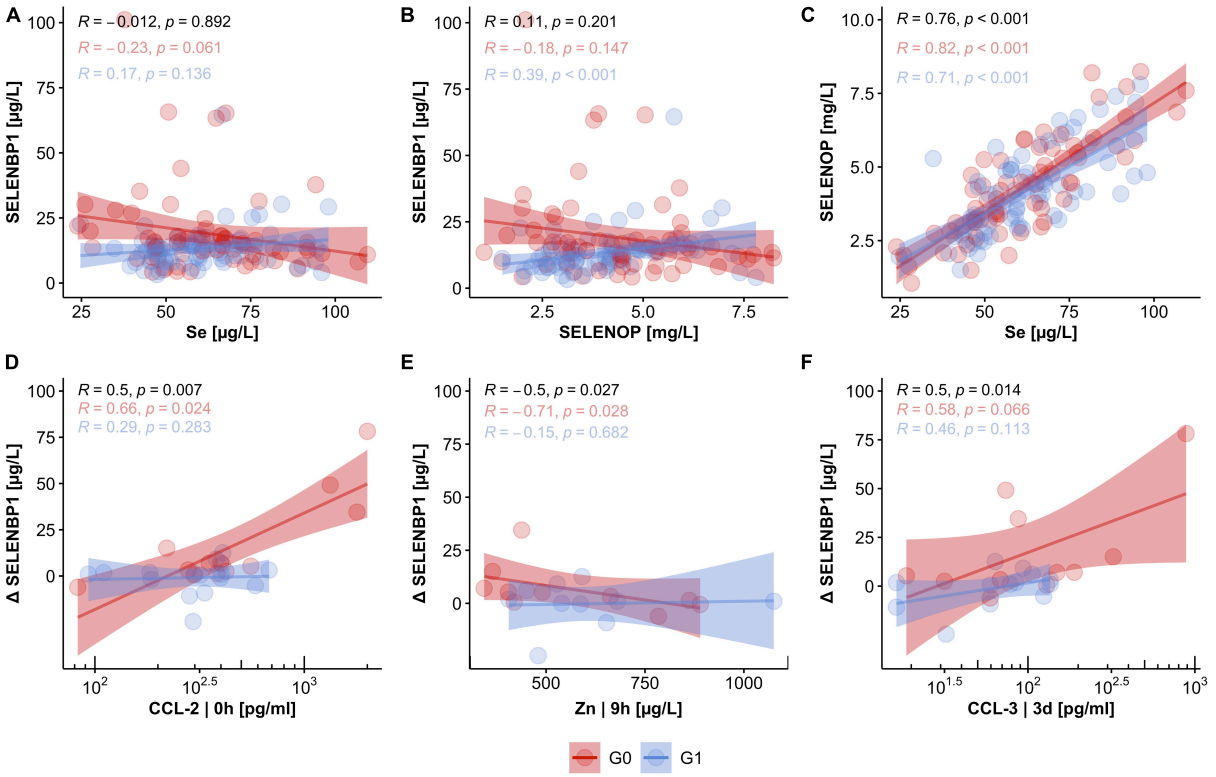


FIGURE 5 | Relation of circulating SELENBP1 concentrations to established biomarkers of Se status and potential covariates in TSCI. The concentrations of circulating SELENBP1 were not associated with either serum Se levels **(A)** or SELENOP concentrations **(B)**, whereas a tight linear correlation between total serum Se and SELENOP concentrations was given **(C)**. Concentrations of CCL-2, Zn, and CCL-3 were strongly associated with declining serum SELENBP1 in patients without neurological improvement after 3 months. In contrast, no significant coherence of CCL-2, Zn, and CCL-3 concentrations with SELENBP1 were observed in the group of patients with neurological improvement in G1 **(D–F)**. Correlation analysis by Pearson, R indicates the correlation coefficient, and p indicates significance, provided on top of the graphics for all samples (black), and the two groups of patients separately (G0; red, G1; blue).

of AIS A, but with SELENBP1 below this threshold showed a high chance for recovery. Using this SELENBP1 threshold, a prediction for remission was enabled with a sensitivity of 98.7% and an odds ratio of 10.4, i.e., with a diagnostically valuable and acceptable degree of reliability.

Due to the observational nature of this study, causal interrelationships cannot be deduced. However, some knowledge of SELENBP1 is available from prior studies. SELENBP1 constitutes a highly conserved protein between species, that may be critical for specific physiological functions, potentially including cell differentiation, protein degradation, intra-Golgi vesicular transport, cell motility and redox modulation (Elhodaky and Diamond, 2018). Its expression is strongly affected by hypoxia, as shown in the context of cancer (Huang et al., 2012; Jeong et al., 2014) and cardiovascular research (Kühn et al., 2019; Kuhn-Heid et al., 2019). It would be highly interesting to study whether serum SELENBP1 is related to locally depressed oxygen levels, employing suitable monitoring techniques such as near-infrared spectroscopy (Casha and Christie, 2011; Ryken et al., 2013; Hawryluk et al., 2015). The assumption that increased SELENBP1 is related to hypoxia and cell death resulting from increased ischemia in TSCI is further supported by the positive correlation with CCL-2 (R = 0.66), that is known as a hypoxia-responsive cytokine (Mojsilovic-Petrovic et al., 2007). It is also consistent with our prior study (Heller et al., 2017), where patients with no improvement in neurological function initially showed increased CCL-2 levels, with a resulting induction of monocyte migration, monocyte proliferation and differentiation (Kiguchi et al., 2010).

Previous studies indicated that peripheral trace element dynamics and concentration changes in the trace element biomarkers are associated with the clinical outcome after TSCI (Heller et al., 2019, 2020; Sperl et al., 2019; Seelig et al., 2020). It was thus hypothesized that there might be a close correlation between serum Se, SELENOP and SELENBP1 concentrations. Unexpectedly, no significant interrelation between SELENBP1 and the other Se status biomarkers was observed, neither in the group with non-remission nor in the remission group. This result highlights that SELENBP1 may not directly affect extracellular serum Se status, potentially due to its relatively low serum concentrations and the different origins of these proteins (mainly liver in case of SELENOP, kidney in case of GPX3 vs. damaged tissue in case of SELENBP1).

Due to the divergent degrees of injuries within AIS classes from A to E, SELENBP1 concentration dynamics may provide

a direct insight into the individual burden of hypoxic stress on a cellular level. Combined with additional diagnostic parameters, SELENBP1 monitoring might pave the way for a more detailed and quantitative clinical assessment strategy after TSCI, thereby supporting the established INSCCI examinations.

Future studies are required to test for a correlation of SELENBP1 dynamics with intraspinal pressure (Phang et al., 2015; Chen et al., 2018; Saadoun and Papadopoulos, 2020), and local tissue oxygenation at the injury site (Kurita et al., 2020). In addition to the sensor-derived data, SELENBP1 might also provide information about other tissues that are damaged or at-risk for degeneration, and remote from the sensor. The SELENBP1 concentrations may also reflect other sorts of injury, including micro-bleedings that might not be detectable by current imaging techniques such as MRI or CT scan. The relevance of these processes and their contribution to the global burden of injured neural tissue and the neuroinflammatory signaling in the second phase after TSCI still need to be evaluated. Recent findings support the importance of spinal cord perfusion pressure (SCPP) monitoring with respect to metabolic characteristics of the injured tissue concerning the chances of neurological remission after TSCI. The data indicate a close correlation between the individual SCPP and metabolic profiles at the injury site, estimated via tissue glucose, lactate, pyruvate, glutamate and glycerol by surface microdialysis (Saadoun and Papadopoulos, 2020). This information might support the identification of individuals with lower potential for remission, and aid in personalized therapy.

With a better characterization of the regulation and function of SELENBP1 in these tissues, its potential role in diseases such as TSCI may be better understood, and SELENBP1 may become a novel and valuable biomarker for diagnostic, monitoring, and prognostic purposes in acute and potentially also in chronic injuries.

Limitations

Despite the relevant and convincingly strong interrelations identified, the current study is not free from limitations. The sample size was relatively small, yet it was sufficient to deduce a cut-off for the early detection of patients with a high chance of neurological remission after TSCI by serum SELENBP1. Still, serum trace elements and their protein biomarkers may be surrogate markers, not necessarily affecting disease course directly or reliably reflecting the physiologically relevant intracellular trace element concentrations (Maret and Sandstead, 2006). Furthermore, the data are from an observational study, and are thus not suitable for deducing mechanistic insights. Finally, the pathophysiological and clinical heterogeneity within the AIS groups complicates the interpretation of the results and necessitates an independent verification of this newly identified biomarker in TSCI.

CONCLUSION

Our results indicate that the analysis of SELENBP1 concentrations in serum provides promising insights regarding the early assessment of both the injury severity after TSCI in AIS A vs. B, C, or D and the individual chance of neurological remission. Monitoring serum SELENBP1 concentrations could assist clinicians in the initial assessment of patients after TSCI, especially in estimating the remission potential of severely injured patients classified as AIS A. Our results support the notion that SELENBP1 constitutes a promising marker for identifying and assessing cell damage and injury, and this potential should be investigated further in the context of other traumatic or degenerative diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethics Committee of the University of Heidelberg (S514/2011). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RH, VD, AM, LS, and BB: conceptualization. JS, RH, QS, JH, LS, and BB: methodology. JS, RH, HC, and LS: software. JS, RH, PH, QS, GK, JH, HC, VD, AM, LS, and BB: visualization and data curation. JS, RH, VD, AM, LS, and BB: validation. JS, RH, HC, VD, AM, LS, and BB: formal analysis. VD, AM, LS, and BB: resources, supervision, and funding acquisition. JS, RH, LS, and BB: writing—original draft preparation. PH, QS, GK, JH, HC, VD, and AM: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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The Type 3 Deiodinase Is a Critical Modulator of Thyroid Hormone Sensitivity in the Fetal Brain

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Thyroid hormones (TH) are critical for the development and function of the central nervous system (CNS). Although their effects on the rodent brain peak within 2–3 weeks postnatally, the fetal brain has been found largely insensitive to exogenously administrated TH. To address this issue, here we examined gene expression in brains from mouse fetuses deficient in the type 3 deiodinase (DIO3), the selenoenzyme responsible for clearing TH. At embryonic day E18.5 qPCR determinations indicated a marked increase in the mRNA expression of T3-responsive genes *Klf9* and *Nrgn*. The increased expression of these genes was confirmed by *in situ* hydridization in multiple areas of the cortex and in the striatum. RNA sequencing revealed 246 genes differentially expressed (70% up-regulated) in the brain of E18.5 *Dio3−/−* male fetuses. Differential expression of 13 of these genes was confirmed in an extended set of samples that included females. Pathway analyses of differentially expressed genes indicated enrichment in glycolysis and signaling related to axonal guidance, synaptogenesis and hypoxia inducible factor alpha. Additional RNA sequencing identified 588 genes differentially expressed (35% up-regulated) in the brain of E13.5 *Dio3−/−* male fetuses. Differential expression of 13 of these genes, including *Klf9, Hr,* and *Mgp*, was confirmed in an extended set of samples including females. Although pathway analyses of differentially expressed genes at E13.5 also revealed significant enrichment in axonal guidance and synaptogenesis signaling, top enrichment was found for functions related to the cell cycle, aryl hydrocarbon receptor signaling, PCP and kinetochore metaphase signaling pathways and mitotic roles of polo-like kinase. Differential expression at E13.5 was confirmed by qPCR for additional genes related to collagen and extracellular matrix and for selected transcription factors. Overall, our results demonstrate that the rodent fetal brain is sensitive to TH as early as E13.5 of gestational age, and suggest that TH distinctly affects brain developmental programs in early and late gestation. We conclude that DIO3 function is critical to ensure an adequate timing for TH action in the developing brain and is probably the main factor underlying the lack of effects on the fetal brain observed in previous studies after TH administration.

Keywords: type 3 deiodinase, *Dio3*, thyroid hormone, brain development, *Klf9*, *Nrgn*

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INTRODUCTION

Thyroid hormones (TH) regulate the expression of a large number of genes in the developing brain, impacting the proliferation, migration and differentiation of multiple brain cell types, and ultimately exerting profound functional effects on the adult CNS (Legrand, 1984; Bernal and Nunez, 1995). Their action is largely mediated by 3,5,3′-triiodothyronine (T3), which can regulate gene transcription upon binding to its nuclear receptor, a DNA-binding transcription factor (Forrest and Visser, 2013). Processes critical for brain maturation such as neurogenesis, neuronal migration and maturation (Richard et al., 2020), dendrite formation, myelination and synaptogenesis are strongly regulated by TH (Bernal and Nunez, 1995; Bernal, 2005). In humans, reduced brain availability of TH during development leads to neurological abnormalities and, in extreme cases, to cretinism and Allan-Herndon-Dudley syndromes, which are characterized by severe intellectual disability and motor deficits (Legrand, 1984; Dumitrescu et al., 2004; Friesema et al., 2004). The importance of TH for the central nervous system is further underscored by the CNS abnormalities noted by studies on animal models with genetic alterations in genes regulating brain TH availability and action. Thus, broad neurological, sensory and behavioral phenotypes are noted in mice with deficits in TH receptors (Dellovade et al., 2000; Venero et al., 2005; Siesser et al., 2006; Wilcoxon et al., 2007; Morgan et al., 2013; Buras et al., 2014; Richard et al., 2017), TH transporters (Friesema et al., 2005; Mayerl et al., 2014; Bernal et al., 2015; Groeneweg et al., 2020) and TH deiodinases (Ng et al., 2009, 2010, 2017; Bocco et al., 2016; Stohn et al., 2016, 2018).

In the rodent, it is during the second and third week of life (equivalent to last trimester of gestation in humans) when the brain exhibits most responsiveness to TH (Bernal, 2005). This time coincides with the differentiation of oligodendrocytes and myelination, as well as with peak levels of THs in the serum due to the maturation of the hypothalamic-pituitary thyroid (HPT) axis (Dussault and Labrie, 1975). It also coincides with peak expression of DIO2 in the brain (Bates et al., 1999; Hernandez et al., 2006), the enzyme that enhances TH action by converting thyroxine (T4) into T3, the hormone with highest affinity for the thyroid hormone nuclear receptor (Bianco et al., 2002; St Germain et al., 2009).

However, earlier in development and especially during rodent fetal life, serum TH levels are much lower than in the adult (Dussault and Labrie, 1975). This is due both to the fact that the HPT axis has not attained full functionality and that the fetal tissues and the utero-placental unit express high levels of the type 3 deiodinase (DIO3) (Galton et al., 1999; Huang et al., 2003), the selenoenzyme that clears T4 and T3 by converting them into metabolites with no significant affinity for the nuclear receptor (Bianco et al., 2002; Hernandez, 2005). The low levels of TH during fetal life and the effects of maternal thyroid status on fetal brain development (Richard and Flamant, 2018) have prompted investigators to assess if the fetal brain is responsive to TH. However, the administration of exogenous TH (either T3 or T4) to rat pregnant dams failed to produce responses of two T3 target genes in the embryonic day 21 (E21) brain (Schwartz et al., 1997). In another study, the administration of T3 to hypothyroid rat dams did not regulate three selected responsive genes in the cerebral cortex at fetal ages E17 and E21, while T4 administration exerted a significant effect (Grijota-Martínez et al., 2011). The lack of response to T3 was noted despite abundant expression of TH transporters and receptors. The authors of these studies reached a similar conclusion and suggested the existence of yet unidentified factors that suppress precocious response to T3. These factors may be involved in T3 signaling or in limiting the amount of T3 that reaches target cells (Schwartz et al., 1997; Grijota-Martínez et al., 2011).

We propose that DIO3 is one of such critical factors. Here we used gene expression profiling in DIO3-deficient mice to show that E18.5 *Dio3*−/− fetal brains exhibit significant and broad changes in gene expression. Our results further show that the fetal brain is responsive to TH as early as E13.5 of gestational age and, in the context of previous work, underscore an important role for *Dio3* in protecting the developing brain from premature T3 action.

MATERIALS AND METHODS

Experimental Animals

Dio3−/− mice have been previously described (Hernandez et al., 2006). Mice used in the present studies were on a C57Bl/6J genetic background. Original female mice on a 129/SVJ genetic background and heterozygous for the *Dio3* inactivating mutation were mated with wild type C57Bl/6J males for seven generations, and the colony was then maintained for more than 26 generations by interbreeding, and by matings of heterozygous females with commercially obtained C57Bl/6J males every 2–3 years to refresh the colony. Experimental mice used in the present study were *Dio3*+/+ and *Dio3*−/− littermate fetuses generated by timed matings of *Dio3* +/− mice. The morning after mating was considered gestational day E0.5. Dams were euthanized using carbon dioxide asphyxiation at embryonic day 13.5 (E13.5) or E18.5. Uterine horns were placed on iced saline and fetal brains were harvested, frozen on dry ice and kept at −80°C until later processing. Mouse studies were approved by the Institutional Animal Care and Use Committee at Maine Medical Center Research Institute.

Fetal Brain RNA Sequencing

We performed RNA sequencing on two batches of total RNA samples from whole brains from individual littermate male fetuses at E13.5 (n = 4, 2, respectively, for *Dio3* + / + and *Dio3*−/− mice) and E18.5 days of embryonic age (n = 3, 5, respectively for *Dio3* + / + and *Dio3*−/− mice). The first batch was submitted to Cofactor Genomics (St. Louis, MO, United States) and sequenced in an Illumina platform. Briefly, rRNA-probes (Ribo-Zero, Epicenter, Madison, WI, United States) were hybridized to total RNA for removal of ribosomal RNA from the sample. Ribo-depleted RNA was then fragmented prior to cDNA synthesis using random primers. Double-stranded cDNA was end-repaired and A-tailed to prepare for adaptor ligation. Indexed adaptors were ligated

to DNA, and the adaptor-ligated DNA was amplified by PCR. Library size and quality was assessed on an Agilent Bioanalyzer and library yield was quantified by qPCR using the KAPA Biosystems Library Quantification kit (Kapa Biosystems, Inc., Wilmington, MA, United States) prior to sequencing on (single end, 75 bp fragment size) on the Illumina HiSeq 2000. The number of aligned reads per sample varied between 40 and 50 million, and represented ∼78% of the total reads per sample. Raw sequence data in Fastq format were assessed for quality (FastQC, [1]) and ribosomal RNA content. Fastq files and processed files for this experiment have been deposited on the Gene Expression Omnibus (GEO) database (Accession number GSE172000). Libraries for a second batch of samples were prepared using a NEBNext rRNA Depletion Kit (New England Biolabs, #6310) to deplete ribosomal RNA from the total RNA. Then, a NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, #E7770) was used to construct the RNA-seq library. The quality and quantity of input RNA and the libraries were assessed using an Agilent Bioanalyzer and Qubit. The multiplex libraries were sequenced (single end, 75 bp fragment size) on a Next Gen 550 at Tufts University Genomics Core Facility.

RNA Sequencing Data Analyses

FASTQC (see text footnote 1) and multiQC v1.9 (Ewels et al., 2016) were used to determine the quality of sequencing data for all samples. Adaptor sequences were trimmed using Cutadapt 3.1 with Python 3.6.2 and the -m 1 option ([2]STAR) (Spliced Transcripts Alignment to a Reference, version 2.5.3a) (Dobin et al., 2013) was used to align the reads to the reference genome (GRCm38/mm10) using the default settings, and the genome index was built based on the GENCODE VM22 annotation (GRCm38/mm10). rRNA and tRNA were filtered using bedtools version 2.26.0, using the GTF files downloaded from the UCSC Table Browser (GRCm38/mm10). HTSeq (Anders et al., 2015) was used to count the reads per transcript (HTseq counts) with a default setting over the union with -s *no* option. For differential gene expression analysis, DESeq2 version 1.30.1 (Love et al., 2014) was performed in the R environment (Version 4.0.3), using the HTseq counts and the significance cutoff set by default to an adjusted $P < 0.05$. ComBat-seq (Zhang et al., 2020) was utilized to adjust for batch effects between the Cofactor Genomics and Tufts University sequencing datasets. Heatmap, MA, PCA, UpSet (Conway et al., 2017) plots were created in R, based on both coding and non-coding transcripts. Functional ontology and pathway analyses of differentially expressed genes were performed using Ingenuity Pathway Analysis (IPA) software (Qiagen, Valencia, CA, United States), and the Database for Annotation, Visualization and Integrated Discovery (DAVID)[3].

[1]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]https://doi.org/10.14806/ej.17.1.200

[3]https://david.ncifcrf.gov/

Real Time Quantitative PCR

Fetal brains were harvested and subsequently frozen on dry ice, and total RNA was extracted using the RNeasy kit from Qiagen (Valencia, CA, United States). Total RNA (1 μg) was reverse transcribed with M-MLV reverse transcriptase in the presence of random decamers (both from Thermo Fisher Scientific, Waltham, MA, United States) at 65°C for 5 min, then 37°C for 50 min. The 20 μl reverse transcription reactions were diluted by adding 230 μl DNase and RNase free water. An aliquot of each sample was mixed together for an internal standard and diluted fourfold. Real-time PCR reactions were set up in duplicate with gene-specific primers and SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, MA, United States) and run on the CFX Connect from Bio-Rad (Hercules, CA, United States), where they underwent an initial 10 min denaturing step, followed by 36 cycles of a denaturing step (94°C for 30 s) and an annealing/extension step (60°C for 1 min). For each individual sample, expression was corrected by the expression of control, housekeeping genes (*Gapdh* or *Rn18s)*, which did not exhibit any significant difference in expression between genotypes. Expression data are shown in arbitrary units and represented as fold-increase over the mean value in the control group. The sequences of the primers used for each gene are shown in **Supplementary Table 1**.

RNAscope *in situ* Hybridization

The heads of E18.5 fetuses were harvested as described above and fixed in 4% formaldehyde for 48 h. After fixation they were paraffin-embedded and cut in five microns coronal or rostro-caudal sections. *In situ* hybridization of *Klf9* and *Nrgn* mRNAs was performed in selected sections of two animals per genotype utilizing the RNAscope technique (Advanced Cell Diagnostics, BioTechne Corporation, Newark, CA, United States) following the manufacturer's suggested procedures. We used the RNAscope Mm-Klf9 and Mm-Nrgn probes (catalog numbers 488371 and 499441, respectively) and the ACD 2.5HD Detection kit (RED). As a negative control, we used the bacterial probe DapB supplied by the manufacturer. Some tissue sections were counterstained with hematoxyline and mounted with EcoMount (catalog # EM897L, Biocare Medical, Pacheco, CA, United States), while other sections were mounted with DAPI Fluoromount-G (Catalog # 0100-20, Southern Biotech, Birmingham, AL, United States). Bright field or fluorescent images of the mRNA signal were taken, respectively, with a Zeiss Axioskop 40 microscope or a Leica SP8 confocal microscope utilizing LAS X software. For anatomic reference, adjacent tissue sections were stained with H&E at our Histology Core facility following standard procedures.

Statistical Analyses

Statistical analysis of data other than RNA-sequencing data was performed using the statistical tools of GraphPad Prism 6 (GraphPad Software, Inc.). A Student's *t*-test, and one-way ANOVA or two-way ANOVA followed by Tukey's test were used to determine statistical significance, which was defined as

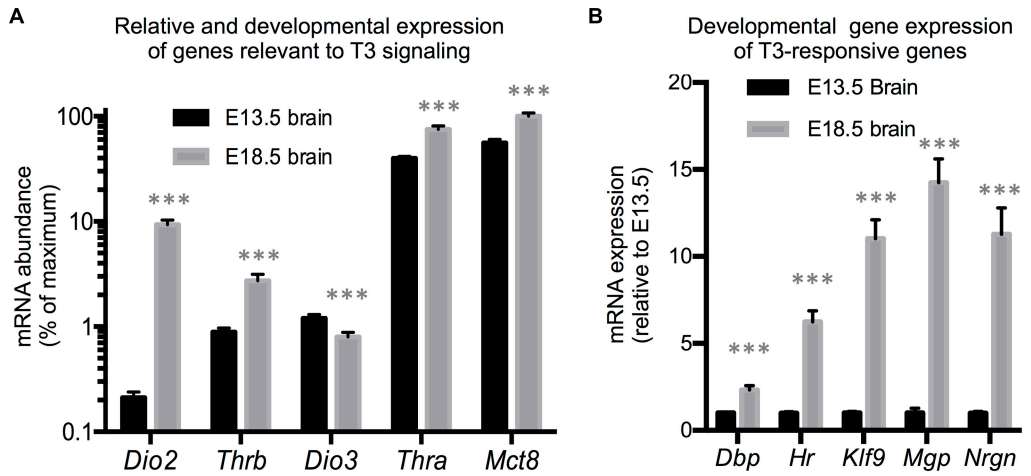

FIGURE 1 | E13.5 and E18.5 brain gene expression. **(A)** Developmental brain expression of genes that regulate T3 action. The relative abundance of mRNA between different genes is an estimation based on CT values on the assumption of similar annealing efficiency of primers used in real time qPCR. **(B)** Developmental brain gene expression of selected T3-responsive genes. Data represent the mean ± SEM of **(A)** eight different brains per experimental group representing both sexes and five different litters or **(B)** 8 (E13.5) and 11 (E18.5) different brains representing mice of both sexes and 6 (E13.5) or 8 (E18.5) litters. ***$P < 0.001$ E13.5 vs E18.5 as determined by the Student's *t*-test.

$P < 0.05$. Significance between different distribution frequencies of genes was determined using a standard chi-square test.

RESULTS

Expression of Genes Related to Thyroid Hormone Action in the Fetal Brain

We first utilized RNA samples isolated from wild type brains from fetuses at E13.5 and E18.5 of gestational age to evaluate the expression of genes with a prominent role in determining TH action. Assuming comparable efficiency in the primers used for each gene, the most abundant mRNAs in E13.5 brains were estimated to be those encoding for the preferred T3 transporter MCT8 (*Slc16a2*) and for TH receptor alpha 1 isoform (THRA1) (**Figure 1A**). Notably less abundant were *Dio3* and TH receptor beta (*Thrb1* isoform) mRNAs, while *Dio2* mRNA was the least abundant. Between E13.5 and E18.5 of gestational age, brain expression of *Dio3* significantly decreased, while the expression of *Mct8*, *Thra,* and *Thrb* was increased, with *Mct8* and *Thra* mRNAs still being the most abundant (**Figure 1A**). Particularly notable was the 50-fold developmental increase in *Dio2* mRNA abundance. The developmental increases in T3 transporter, receptors and T3-generating DIO2 enzyme suggest that the E18.5 brain should exhibit increased T3 signaling compared to the E13.5 brain. We thus measured the expression of five well-established T3-responsive genes in the brain (Chatonnet et al., 2015) at both developmental ages. We observed significant developmental increases in the expression of Krüppel-like factor 9 (*Klf9*), hairless (*Hr*), Neurogranin (*Nrgn*), D site albumin promoter binding protein (*Dbp*) and Matrix gla protein (*Mgp*) (**Figure 1B**), supporting the hypothesis that the mechanisms controlling T3 action are more mature in the E18.5 brain than earlier in gestation.

Expression of *Klf9* and *Nrgn* in the *Dio3*−/− E18.5 Brain

Based on the data above, and since we have previously shown that serum T3 is elevated in *Dio3*−/− fetuses late in gestation (Hernandez et al., 2006), we focused on E18.5 developmental stage to evaluate T3-dependent gene expression in the *Dio3*−/− brain. We chose *Klf9* and *Nrgn* for these studies. Real time qPCR analysis of RNA from whole E18.5 *Dio3*−/− brains indicated a more than threefold increase in *Klf9* expression when compared with that of *Dio3*+/+ littermates (**Figure 2A**). We observed no indication of sexual dimorphisms in *Klf9* expression in either *Dio3*+/+ or *Dio3*−/− fetuses. *In situ* hybridization using RNAscope revealed that the increase is apparent in most cortical and striatal areas (**Figure 2B**). *Klf9* was strongly expressed in the neocortex, except for the most external layer (**Figure 2C**). Compared to *Dio3*+/+ littermates, the *Dio3*−/− brain exhibited robust increases in *Klf9* expression across multiple areas of the neocortex (**Figure 2C**), including the motor and sensory cortices (**Figure 2D**) and both external and deeper cortical layers in which *Klf9* was expressed (**Figures 2E,F**, respectively). Marked increases in *Klf9* mRNA were also observed in the *Dio3*−/− septum and striatum (**Supplementary Figures 1B,C**, respectively). Elevated *Klf9* mRNA was more modest in the periventricular zone of the third ventricle (**Supplementary Figures 1E,F**). Interestingly, no apparent changes in *Klf9* expression were observed in the periventricular zone of the lateral ventricles (**Figure 2G** and **Supplementary Figure 1C**).

The expression of *Nrgn* was also significantly elevated in the E18.5 *Dio3*−/− brains compared to that of littermates, both in males and females (**Figure 3A**). *In situ* hybridization indicated

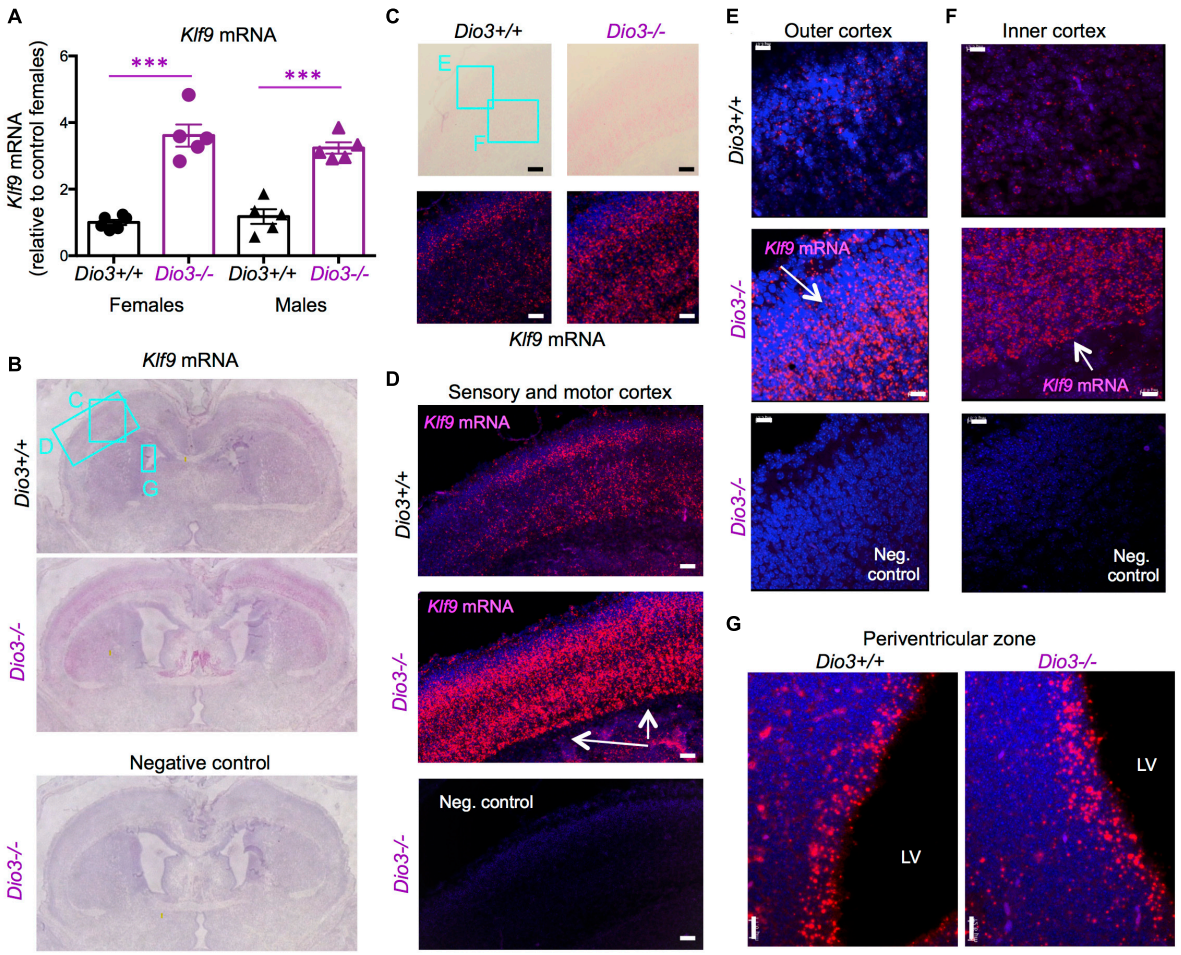

FIGURE 2 | *Klf9* expression is elevated in *Dio3*−/− brains. **(A)** *Klf9* mRNA expression in whole E18.5 brains. Data represent the mean ± SEM of 11 and 10 different samples from E18.5 *Dio3*+/+ and Dio3−/− mice, respectively, divided by sex. ***$p < 0.001$ E13.5 vs E18.5 as determined by the ANOVA and Tukey's *post hoc* test. B-G, Bright field and fluorescent images of *in situ* hydridization of *Klf9* mRNA in whole brain coronal sections **(B)**, motor cortex **(C)**, motor and sensory cortex **(D)**, outer and inner cortical layers [**(E,F)**, respectively] and lateral periventricular zone **(G)**. Images are representative of two different animals of each genotype. Rectangles and letters indicate the panels in which those anatomic regions are amplified. St, striatum; Lv, lateral ventricle. Negative control was hybridized with a bacterial probe. Arrows indicate major areas of differential expression. Scale bars, 45, 15, and 12 microns for panels **(C–G)**, respectively.

elevated *Nrgn* expression in most brain regions (**Figure 3B** and **Supplementary Figure 2A**). *Nrgn* expression increase in *Dio3*−/− fetuses was most dramatic in the frontal/cingular cortex (**Figure 3D** and **Supplementary Figure 2B**) as well as in the motor cortex (**Figure 3C**) and striatum (**Figure 3F**). *Nrgn* expression was also elevated in the motor cortex (**Figure 3G**), but no appreciable change was observed in the piriform cortex (**Figure 3E**). Similarly to *Klf9*, no *Nrgn* expression was noted in the most outer layer of the cortex (**Supplementary Figure 2B**). The hematoxylin counterstaining and the pattern of cortical *Nrgn* expression suggested increased brain cortical thickness in *Dio3*−/− fetuses (**Supplementary Figure 2B**).

Gene Expression Profiling of the *Dio3*−/− Fetal Brain

Results on *Klf9* and *Nrgn* expression suggested that the E18.5 brain is sensitive to TH and that mouse DIO3 deficiency is an excellent model to probe T3-dependent gene expression in the early development of the brain. Thus, we used RNA sequencing to perform a gene expression profiling of three *Dio3*+/+ and five *Dio3*−/− male brains at E18.5 of embryonic age. Principal component analysis (PCA) distinctively separated *Dio3*+/+ and *Dio3*−/− samples along PC1 (34% variance) but not along PC2 (28% variance) (**Figure 4C**). One of the *Dio3*+/+ samples clustered within the *Dio3*−/− samples (**Figure 4A**). We identified 246 differentially expressed genes (DEGs) with an adjusted $P < 0.05$ that are represented in MA and volcano plots in **Figure 4** (**Figures 4B,D**, respectively). [599 genes were differentially expressed based on a non-adjusted $P < 0.01$ (**Supplementary Data**)]. DEGs showed a marked bias toward up-regulation, as 171 genes (70% of DEGs at that statistical threshold) (**Figure 4D**). Differential expression of some DEGs was confirmed by qPCR using the same plus additional, non-related samples from males and female E18.5 fetuses. Strong

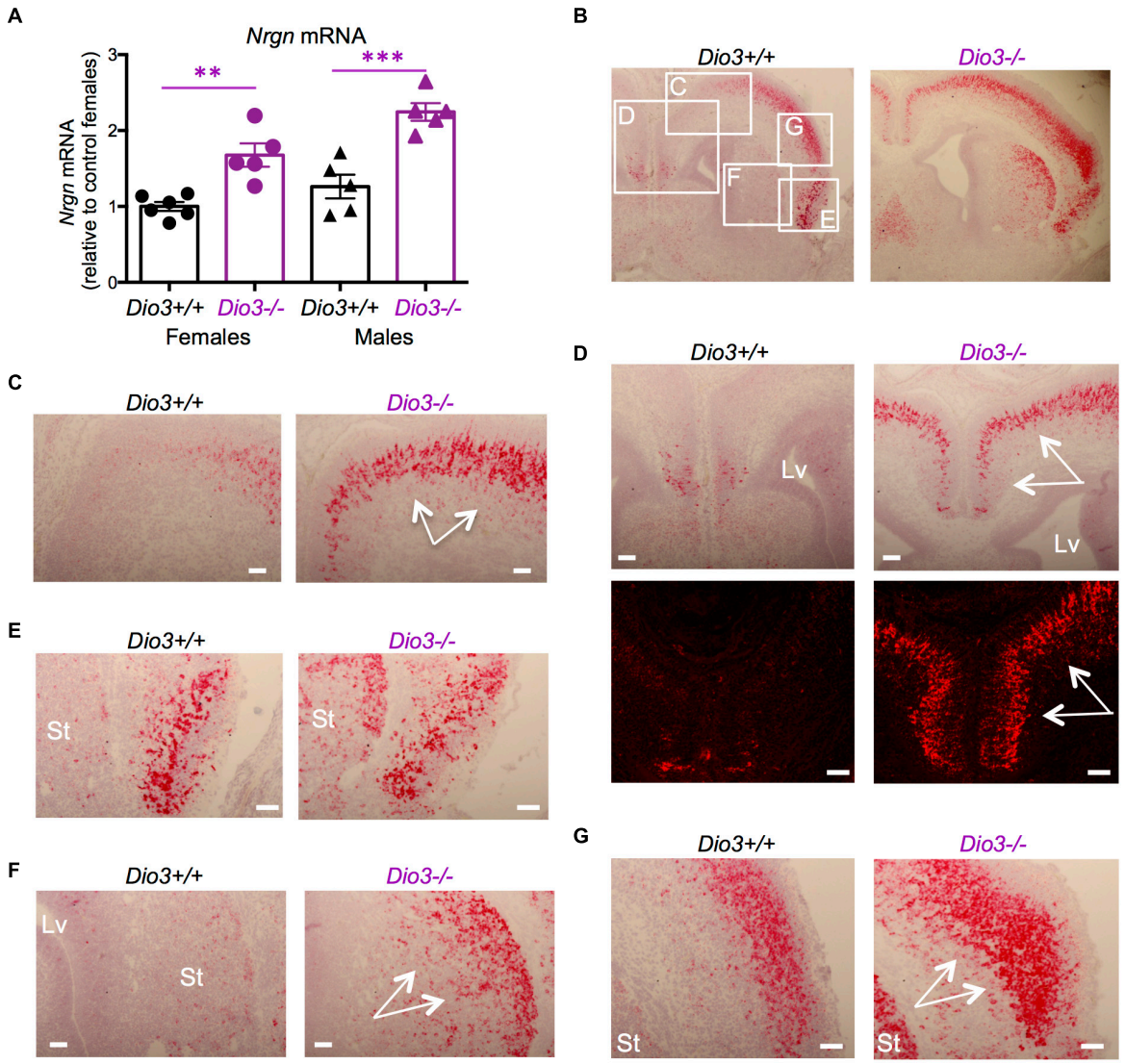

FIGURE 3 | *Nrgn* expression is elevated in E18.5 *Dio3*−/− brains. **(A)** *Nrgn* mRNA expression in whole E18.5 brains. Data represent the mean ± SEM of 11 and 10 different samples from E18.5 Dio3+/+ and Dio3−/− mice, respectively, divided by sex. **and ***$p < 0.01$ and $p < 0.001$, respectively, E13.5 vs E18.5 as determined by the ANOVA and Tukey's *post hoc* test. **(B–G)**, Bright field and fluorescent images of *in situ* hydridization of *Nrgn* mRNA in whole brain coronal sections **(B)**, motor cortex **(C,D)**, frontal/cingular cortex **(D)**, piriform cortex **(E)**, striatum **(F)** and second somatosensory cortex **(G)**. Images are representative of two different animals of each genotype. Rectangles and letters indicate the panels in which those anatomic regions are amplified. St, striatum; Lv, lateral ventricle. Arrows indicate areas of major differential expression. Scale bars are 40 microns.

up-regulation was validated for the expression of *Cplx3, Dio3* itself, *Dio3os, Hr, Mgp,* and *Slc22a2* (**Figure 4E**, left) [please note that *Dio3*−/− mice carry a triple point mutation in *Dio3* that renders the DIO3 enzyme fully inactive, but *Dio3* mRNA is present and detectable in these animals (Hernandez et al., 2006)]. Significant up-regulation was also confirmed for the expression of *Cldn12, Dbp, Gfap, Gpr37l1, Lpl, Sorl1,* and *Vegfa*, while the expression of *Dio2* was modestly repressed (**Figure 4E**, right).

To investigate whether the brain capable of responding to T3 even earlier in development, we also submitted for RNA sequencing E13.5 brain RNA samples from four *Dio3*+/+ and two *Dio3*−/− males. Sample clustering and MA plot highlighting DEGs based on an adjusted $P < 0.05$ are shown in **Figures 5A,B**. PCA robustly separated *Dio3*+/+ and *Dio3*−/− samples along PC1 (69% of variance), but not along PC2 (17% of variance) (**Figure 5C**). Samples of different genotypes clustered separately (**Figure 5A**). At this gestational age, we identified 588 DEGs based on an adjusted $P < 0.05$. At this age, there was a bias toward down-regulation, with 383 (65%) of DEGs being down-regulated (**Figure 5D**). [1,012 differentially expressed based on a non-adjusted $P < 0.01$ (**Supplementary Data**)]. Using the same plus additional RNA samples from female E13.5 brains, we used qPCR and confirmed the differential expression of some genes consistently found to be up-regulated by T3 including *Dbp,*

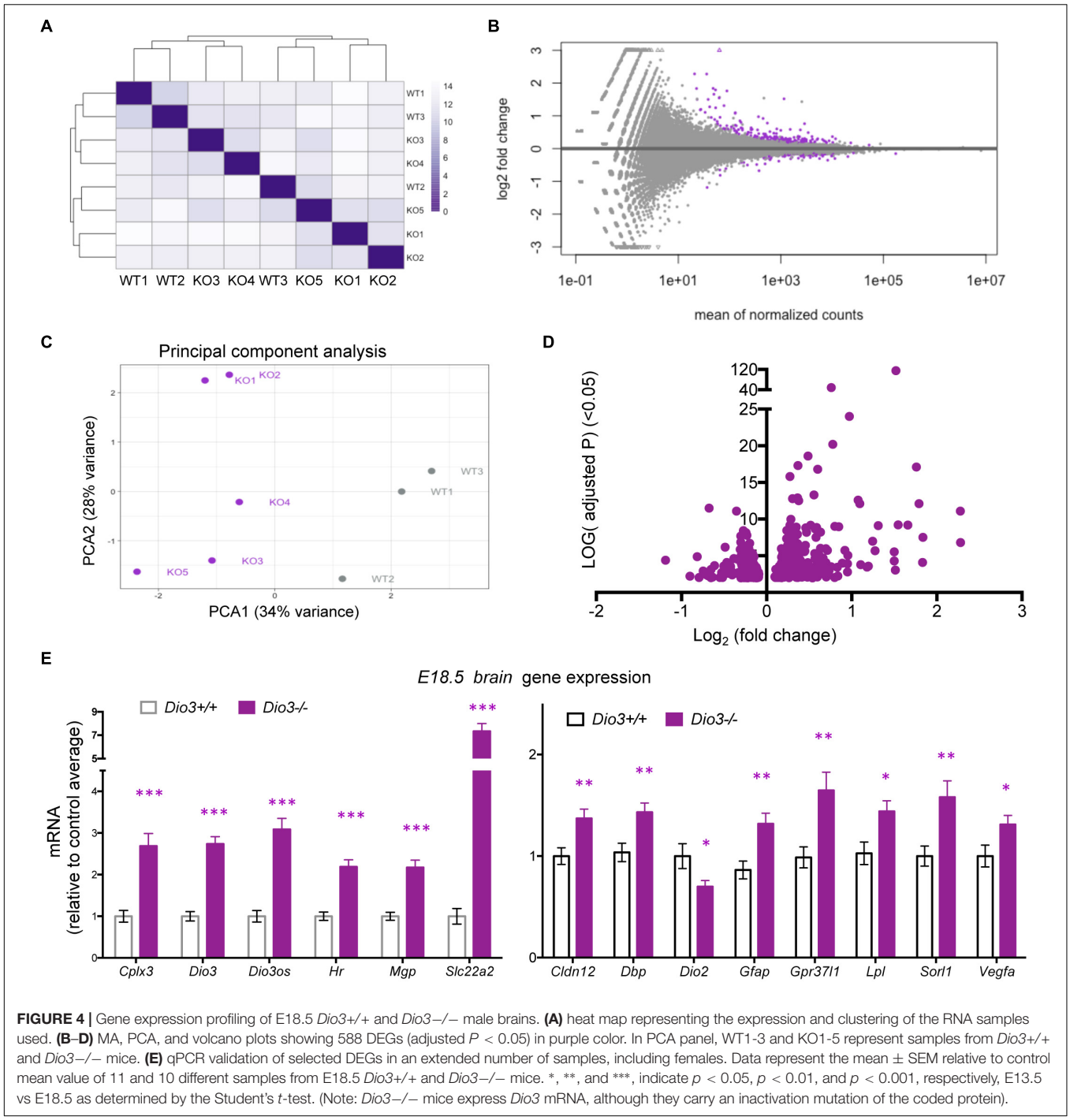

FIGURE 4 | Gene expression profiling of E18.5 *Dio3*+/+ and *Dio3*−/− male brains. **(A)** heat map representing the expression and clustering of the RNA samples used. **(B–D)** MA, PCA, and volcano plots showing 588 DEGs (adjusted $P < 0.05$) in purple color. In PCA panel, WT1-3 and KO1-5 represent samples from *Dio3*+/+ and *Dio3*−/− mice. **(E)** qPCR validation of selected DEGs in an extended number of samples, including females. Data represent the mean ± SEM relative to control mean value of 11 and 10 different samples from E18.5 *Dio3*+/+ and *Dio3*−/− mice. *, **, and ***, indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, E13.5 vs E18.5 as determined by the Student's *t*-test. (Note: *Dio3*−/− mice express *Dio3* mRNA, although they carry an inactivation mutation of the coded protein).

Dio3, Klf9, Mgp, Mme, Sned1, and *Thrb* (**Figure 5E**). We also confirmed up-regulation of genes with important developmental roles including *Igf1, Igf2, H19,* and *Meg3* (**Figure 5E**). Overall, these results show that the fetal brain is sensitive to T3 as early as embryonic age E13.5.

We used DAVID and IPA to analyze the ontology and biological functions of DEGs at each gestational age. To avoid bias due to differences in the number of genes entered into these algorithms, we used 588 DEGs at E13.5 (adjusted $P < 0.05$) and 599 DEGs at E18.5 (non-adjusted $P < 0.01$). For each dataset statistically enriched terms with a FDR < 0.0001 are listed in the **Supplementary Data**. Selected enriched terms identified by DAVID are shown for E13.5 and E18.5 DEGs in **Tables 1**, **2**, respectively. There were enriched biological themes common for both sets of DEGs showing comparable statistical significance, including "neurogenesis," "methylation," extracellular matrix,"

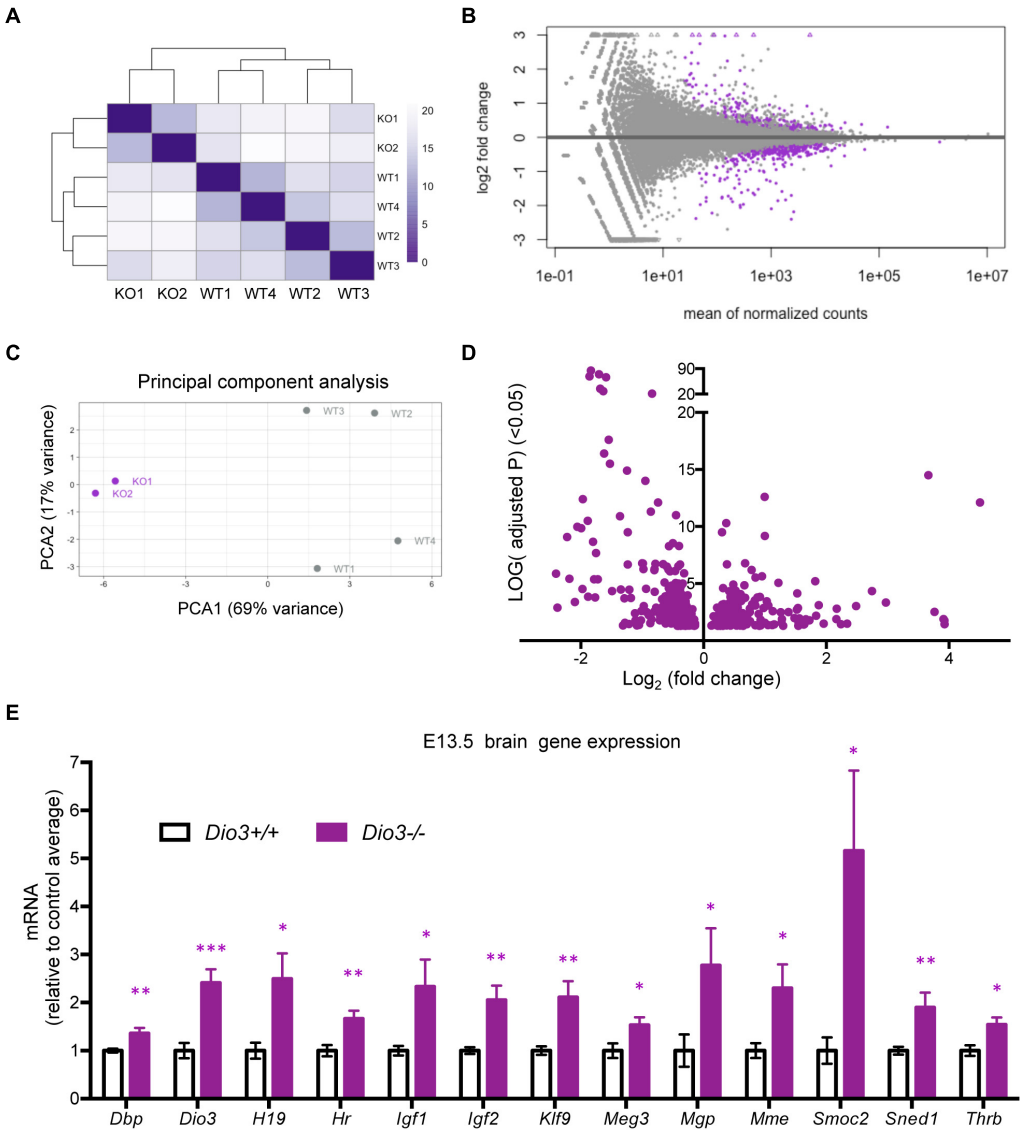

FIGURE 5 | Gene expression profiling of E13.5 *Dio3*+/+ and *Dio3*−/− male brains. **(A)** Heat map representing the expression and clustering of the RNA samples used. **(B–D)** MA, PCA, and volcano plots showing 588 DEGs (adjusted $P < 0.05$) in purple color. In PCA panel, WT1-4 and KO1-2 represent samples from *Dio3*+/+ and *Dio3*−/− mice. **(E)** qPCR validation of selected DEGs in an extended number of samples, including females. Data represent the mean ± SEM relative to control mean value of 8 and 6 different samples from E18.5 *Dio3*+/+ and *Dio3*−/− mice. *, **, and ***, indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, E13.5 vs E18.5 as determined by the Student's *t*-test. (*Dio3*−/− mice express *Dio3* mRNA, although they carry an inactivation mutation of the coded protein).

"alternative splicing," "activator," "repressor," "differentiation." Enrichment terms including "glycoprotein" and "EGF-like" were more significant or specific for DEGs at E18.5. Other enrichment terms were much more significant or specific for DEGs at E13.5, including "phosphoprotein," "developmental protein," "transcriptional regulation," "DNA binding," "nucleus," chromosome," "homeobox," and "cell cycle" (**Tables 1**, **2**).

Ingenuity pathway analysis analysis of the two DEGs datasets indicated enrichment in a number of canonical pathways, some of which were common (**Supplementary Data**). However, the most statistically significant canonical pathways were very different between the two developmental stages (**Table 3**). While E13.5 DEGs were most enriched in pathways related to the cell cycle and nuclear DNA rearrangement during mitosis, E18.5 DEGs showed top enrichment in pathways related axon guidance, synaptogenesis, glycolysis and hypoxia inducible factor (**Table 3** and **Supplementary Data**).

Results from IPA concerning pathway upstream analysis rendered substantially different results. The most significant upstream regulators identified from E13.5 DEGs, including CDKN1A, asparaginase and E2F4, barely showed any significance for E18.5 DEGs, an observation that also applies to the activation Z scores associated with those regulators (**Figure 6A**). The activation Z scores and the effects of these

TABLE 1 | Results of DAVID analysis of DEGs in E13.5 *Dio3−/−* brain.

UP_KEYWORD Term	Fold Enrichment	FDR
DNA-binding*	4.68	5.36E-70
Nucleus	2.52	9.79E-55
Chromosome	7.11	9.43E-36
Developmental protein*	4.32	7.40E-34
Phosphoprotein*	1.69	1.83E-27
Homeobox	7.53	9.16E-27
Nucleosome core	13.49	7.86E-25
Cell cycle	4.38	9.24E-22
Ubl conjugation*	2.91	1.14E-21
Transcription regulation*	2.67	5.50E-21
Transcription*	2.61	2.13E-20
Mitosis	6.35	3.48E-18
Cell division	5.10	2.11E-17
Citrullination	11.21	2.83E-17
Isopeptide bond*	3.05	6.26E-15
DNA replication	10.21	6.26E-15
Acetylation	1.88	1.21E-12
Activator*	3.31	1.07E-11
Methylation*	2.59	8.08E-10
Neurogenesis*	4.44	1.40E-08
Alternative splicing*	1.47	4.55E-07
Extracellular matrix*	4.13	5.19E-07
Differentiation*	2.55	2.97E-06
Repressor*	2.68	6.03E-06
Centromere	5.03	6.50E-06
Cytoskeleton	1.96	8.19E-05
Glycoprotein*	1.44	8.71E-05
Microtubule	3.19	1.58E-04
Zinc	1.59	3.38E-04
Cyclin	8.03	4.10E-04
LIM domain	5.70	4.63E-04
Kinetochore	4.88	6.18E-04
DNA repair	2.87	9.10E-04

*Based on analysis of 588 DEGs based on an adjusted P < 0.05. * indicates a common term for both E13.5 and E18.5 DEGs.*

TABLE 2 | Results of DAVID analysis of DEGs in E18.5 *Dio3−/−* brain.

UP_KEYWORD Term	Fold Enrichment	FDR
Glycoprotein*	2.067362941	8.62E-24
Disulfide bond	1.959425632	2.64E-15
Alternative splicing*	1.650343088	1.29E-12
Phosphoprotein*	1.452714812	1.44E-12
Cell adhesion	3.761449216	9.84E-12
Signal	1.641066106	9.84E-12
Developmental protein*	2.492625787	3.55E-09
EGF-like domain	4.55449827	2.13E-08
Secreted	2.002690132	2.60E-08
Neurogenesis*	4.289256546	2.77E-08
Activator*	2.766835241	9.03E-08
Transcription regulation*	1.875782586	4.01E-07
Transcription*	1.857455826	4.01E-07
DNA-binding*	1.883655912	1.98E-06
Extracellular matrix*	3.840388721	2.89E-06
Methylation*	2.166306228	4.29E-06
Differentiation*	2.429644231	9.75E-06
Cell membrane	1.471844735	1.50E-05
Calcium	2.182566746	2.06E-05
Synapse	2.857724405	7.25E-05
Ubl conjugation*	1.746841555	1.33E-04
Metal-binding	1.444725859	1.61E-04
Repressor*	2.351386027	2.17E-04
Glycolysis	8.968858131	2.55E-04
GPI-anchor	3.923875433	6.20E-04
Membrane	1.206581528	6.20E-04
Cell junction	2.07769501	8.56E-04
Isopeptide bond*	1.852826804	9.98E-04

*Based on analysis of 599 DEGs based on non-adjusted P < 0.01. * indicates a common term for both E13.5 and E18.5 DEGs.*

TABLE 3 | Top canonical pathways (IPA) enriched in E13.5 and E18.5 DEGs.

Canonical Pathway	-LOG(*P* value)	Age
Cell Cycle Control of Chromosomal Replication	12.1	E13.5
Aryl Hydrocarbon Receptor Signaling	8.5	E13.5
PCP pathway	7.15	E13.5
Kinetochore Metaphase Signaling Pathway	7.09	E13.5
Mitotic Roles of Polo-Like Kinase	5.75	E13.5
Wnt/β-catenin Signaling	5.13	E13.5
Axonal Guidance Signaling	8.26	E18.5
HIF1α Signaling	6.56	E18.5
Glycolysis I	5.61	E18.5
Synaptogenesis Signaling Pathway	4.41	E18.5
Hepatic Fibrosis/Hepatic Stellate Cell Activation	3.97	E18.5
TR/RXR Activation	3.63	E18.5

regulators, as well as the main canonical pathways affected as revealed by IPA (**Supplementary Data**) suggest a reduction in cell proliferation, consistent with the results from DAVID. In contrast, a substantial proportion of the top significant upstream pathways affected by DEGs at E18.5 were also affected at E13.5 with comparable significance, including those regulated by beta-estradiol, FGF2, TGFB1, AGT, and tretinoin, a retinoic acid agonist (**Figure 6B**). Interestingly, some of these regulators exhibited similar (tretionein, TGFB1) or opposite (beta-estradiol, FGF2) activation scores at each developmental stage (**Figure 6B**). Furthermore, although the overlap of DEGs at both E13.5 and E18.5 was significant (95 genes of 1,012 and 599, respectively), it was substantially lower than anticipated if we consider the hypothesis that the effects of T3 on the fetal brain are largely comparable at both gestational ages. A large majority of DEGs were not common between E13.5 and E18.5 brains and about half of the common DEGs showed opposite regulation between developmental ages (**Figure 7A**). Using additional samples from female fetuses, we used qPCR to validate the differential expression of E13.5 DEGs. The differential expression of some of them was borderline significant. These DEGs included Arx, *Dlx1, Dlx2, Dlx5, Isl1,* and *Islr2* (**Figure 7B**), which are of importance for neuronal and cortical development. We also confirmed the

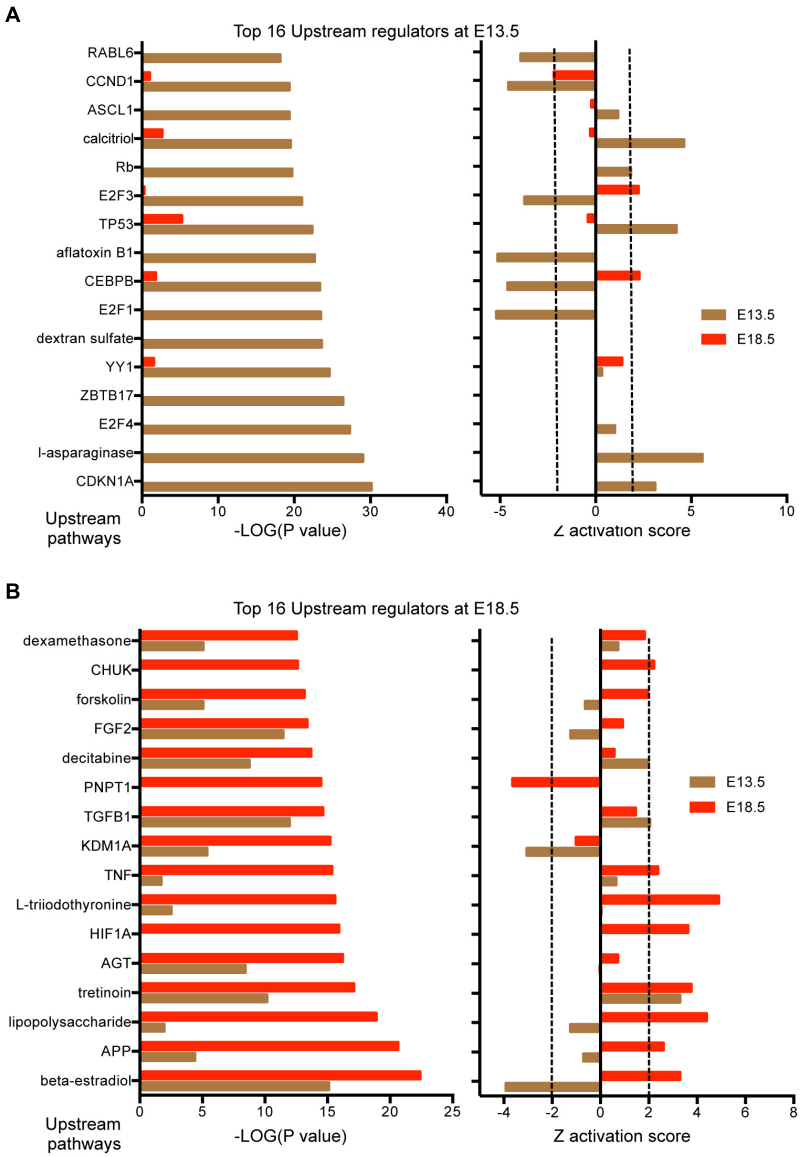

FIGURE 6 | Upstream regulators affected by DEGs in the E13.5 and E18.5 brains of *Dio3*+/+ and Dio3−/− mice. **(A)** Top significant upstream pathways affected in Dio3−/− mice at E13.5 with corresponding statistical values and Z activation scores at E18.5. **(B)** Top significant upstream pathways affected in *Dio3*−/− mice at E18.5 with the corresponding statistical values and Z activation scores at E13.5. Dotted lines indicate the Z score threshold hat IPA considers significant for activation or inactivation of a certain pathway.

differential expression at E13.5 of several genes related to collagen and extracellular matrix formation (**Figure 7C**). Taken together, these observations suggest common biological processes in the brain affected by T3 at both developmental ages, but also indicate that some of them are distinct and specific to E13.5.

An initial analysis of gene expression profiles between developmental ages within the same genotype identified 9,408 DEGs (adjusted $P < 0.01$) in the *Dio3*+/+ brain between E13.5 and E18.5. In *Dio3*−/− mice, 3,841 genes were identified as differentially expressed between developmental ages, the vast majority of them (3,128 genes, 81%) overlapping with those in *Dio3*+/+ mice (**Supplementary Figure 3**). These results indicate that there are more than five thousand genes that showed significant changes in brain expression during development in *Dio3*+/+ mice but not in *Dio3*−/− mice (**Supplementary Figure 3**).

DISCUSSION

The importance of TH for the development of the CNS is well established in mammals, including humans (Legrand, 1984). In rodents, their broader and more profound effects on the regulation of brain gene expression occur in late neonatal life

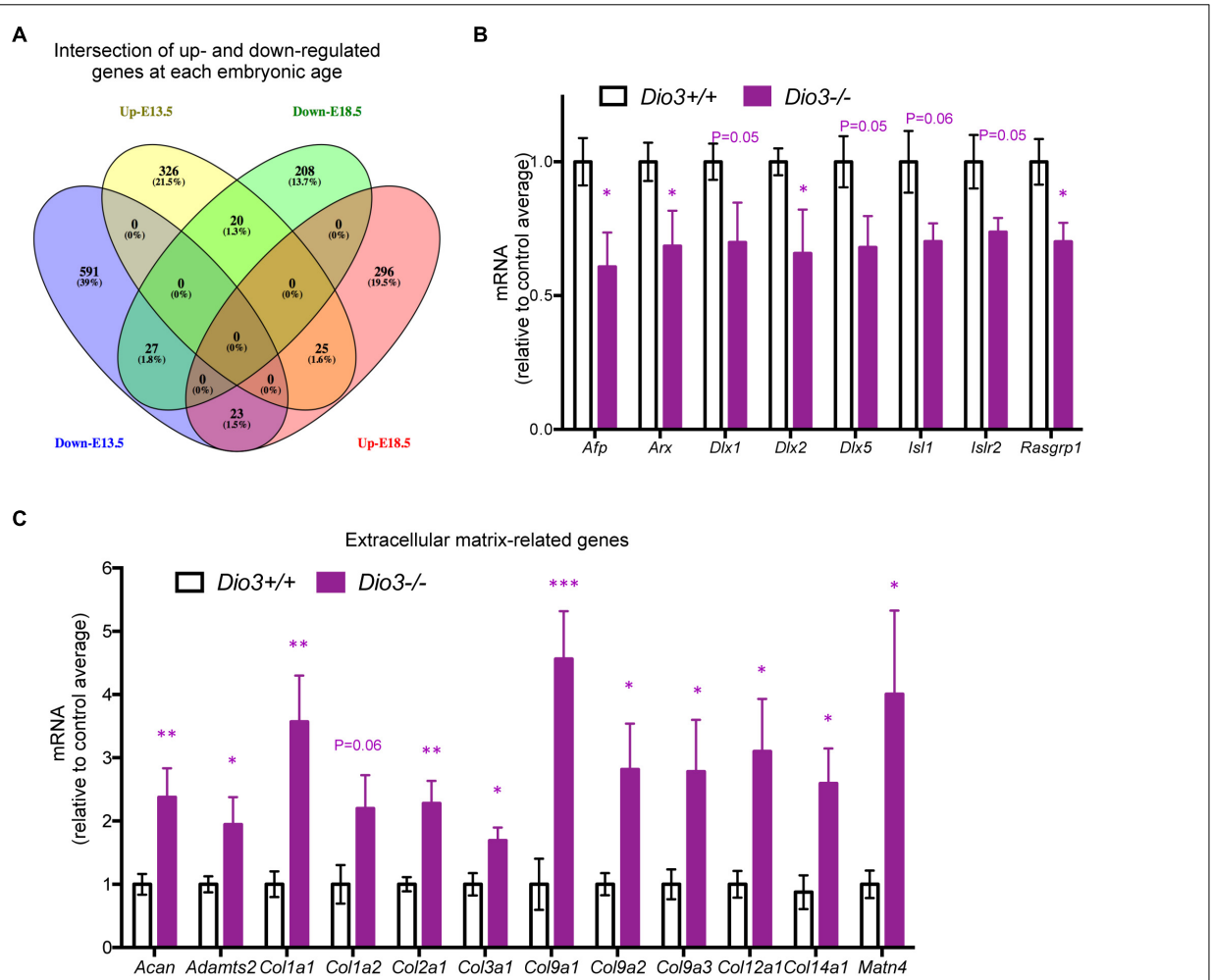

FIGURE 7 | Overlap and differential gene expression in E13.5 Dio3−/− brains. **(A)** Venn diagram of up- and down regulated genes in Dio3−/− brain at E13.5 and E18.5. **(B)** qPCR validation of additional DEGs that are down-regulated in E13.5 *Dio3*−/− brains. **(C)** qPCR validation of differential expression of genes related to extracellular matrix components in E13.5 *Dio3*−/− brains. Data represent the mean ± SEM, relative to control mean value, of 8 and 6 different samples from E18.5 *Dio3*+/+ and *Dio3*−/− mice. *, **, and ***, indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, E13.5 vs E18.5 as determined by the Student's *t*-test.

(Bernal, 2005; Morte et al., 2010; Hernandez et al., 2012), but their actions at earlier developmental stages, especially *in utero*, have remained unclear. The administration of TH to pregnant rodents in late gestation has minimal or negligible effects on the expression of T3-regulated genes (Schwartz et al., 1997; Grijota-Martínez et al., 2011), despite the relatively abundant expression in the fetal brain of the T3 receptor THRA and the main T3-transporter, MCT8 (López-Espíndola et al., 2014; Mayerl et al., 2014). To determine whether the fetal brain is sensitive to T3, here we used a DIO3-deficiency mouse model, in which an excess of T3 in the fetus is produced by impaired T3 clearance.

Developmental expression profiles of genes enhancing brain T3 availability and action, as well as selected genes regulated by T3 (Chatonnet et al., 2015), showed significantly increased expression at E18.5 compared to E13.5, suggesting that components of T3 signaling are more mature at the later fetal age. Using qPCR and *in situ* hydridization we showed that *Dio3*−/− mice exhibited robust mRNA up-regulation of *Klf9* and *Nrgn*, two well-established T3-regulated genes (Martínez de Arrieta et al., 1999; Chatonnet et al., 2015) that have been shown to be regulated in primary culture of fetal neurons (Gil-Ibanez et al., 2014) and in different regions of the neonatal and adult brain (Iñiguez et al., 1992; Martínez de Arrieta et al., 1999). The expression of both *Klf9* and *Nrgn* was increased in most brain regions of E18.5 *Dio3*−/− mice in which they were expressed, predominantly areas of the cortex and striatum, although *Nrgn* manifested region-specific sensitivity to thyroid hormones, as previously described in older animals (Guadaño-Ferraz et al., 1997), with most prominent up-regulation in the frontal cortex and striatum.

Unbiased gene expression profiling by RNA sequencing confirmed the sensitivity of the *Dio3*−/− fetal brain to T3 by identifying, with a limited sample number, several hundred differentially expressed genes. Many of these genes are included in a published compendium of T3 regulated genes in the CNS (Chatonnet et al., 2015), suggesting that T3-regulation of gene expression in the brain at this developmental age

is largely comparable to that later in life in terms of the genes that are regulated. Canonical pathways enriched in DEGs as identified by IPA and DAVID, including axon guidance signaling and synaptogenesis are consistent with known TH effects in the developing brain (Bernal, 2005). Activation of glycolysis-, hypoxia- and inflammation-related pathways (HIF1a and lipopolysaccharide) may reflect the action of T3 excess on oxidative-dependent metabolism and the brain cell response to reduced T3 levels, as HIF1a is known to activate *Dio3* expression in the brain (Simonides et al., 2008). One of the top canonical pathways and upstream regulators identified by IPA as being activated is that of thyroid receptor-retinoid X receptor ("TR-RXR") and "triiodothyronine," providing further confirmation of enhanced T3 signaling. The significant activation of upstream regulators (tretionein, dexamethasone) related to other nuclear receptors also suggest the occurrence of cross-talk between TH signaling and pathways regulated by the retinoic acid and glucocorticoid receptors, something that has been proposed in a model of primary culture of fetal neurons (Gil-Ibanez et al., 2014).

At E13.5, we also observed and validated the increased expression of well-established T3-regulated genes including *Dbp, Dio3, Hr, Klf9, Mgp, Mme, Sned1,* and *Thrb.* This finding indicated that the brain is sensitive to T3 as early as E13.5. However, a large proportion of DEGs at this embryonic age is substantially different from those identified at E18.5, suggesting largely different biological effects. This is illustrated by the rather low overlap in DEGs between both ages, and further confirmed by the different biological terms and pathways identified by DAVID and IPA as achieving top statistical significance. Both DAVID and IPA algorithms suggest E13.5 DEGs been involved in the cell cycle. In particular, upstream regulators identified by IPA indicate negative activation scores of pathways or compounds promoting transcription related to the cell cyle (RABL6, CCDN1, E2F3, E2F1, CEBPB, and aflatoxin) (Leone et al., 1998; Piva et al., 2006; Bryant et al., 2020; Huang et al., 2020), and positive activation scores for pathways opposing cell division (TP53, asparaginase, and CDKN1A) (Scotti et al., 2010; Lüdtke et al., 2013; Fischer et al., 2016; Yang et al., 2017). However, some genes like *E2f3* and *E2f1*, with primary functions regulating the cell cycle also play roles in the migration (McClellan et al., 2007) and apoptosis of neurons (Hou et al., 2000) and in neurogenesis (Cooper-Kuhn et al., 2002). These analyses suggest an effect of T3 in suppressing brain cell proliferation or influencing neuronal homeostasis at this embryonic stage. Since most of the DEGs at this developmental stage do not overlap with known T3-target genes in the CNS (Chatonnet et al., 2015), it is possible that they are not primary targets of T3. The differential expression observed may be secondary to changes in cellular subtypes characteristics and population. It is possible that at this early stage T3 targets a particular cell type that in turn will affect gene expression in other cells in a paracrine manner. This possibility is supported by the increased expression of genes involved in the composition of the extracellular matrix, including several collagen genes.

An interesting observation is that IPA identifies beta-estradiol as an upstream regulator whose pathway is significantly altered at both developmental stages. However, the activation score is completely the opposite, with the beta-estradiol pathways being markedly suppressed at E13.5 and activated at E18.5 in *Dio3*−/− fetuses. Furthermore, in E13.5 brains, the strong statistical significance of the differential expression of some genes (*Dlx1, Dlx2, Dlx5, Arx, and Isl1*) in the RNA sequencing experiment (which used only male samples) was barely achieved in the qPCR determinations, which also included female samples. This raises the possibility of a potential sexually dimorphic effect of T3 on the brain in early development that need further investigations, especially since some of the above genes are critically involved in neuronal specification and cortical development (Pla et al., 1991; Eisenstat et al., 1999; McKinsey et al., 2013; Erb et al., 2017; Zhang et al., 2018). It is interesting to note that genes of the *Dlx* family, which are abnormally expressed in the E13.5 *Dio3*−/− brain, influence the development of interneurons (Long et al., 2009). Some of them will later develop into parvalbumin-positive neurons, a known target of T3 in the mature brain (Sui et al., 2007; Mittag et al., 2013; Bastian et al., 2014), suggesting a thyroid hormone developmental programming of the adult brain in terms of T3 responsiveness and T3-dependent brain functions.

Despite the DEGs identified at both gestational ages as regulated by T3, their number is modest compared to those that are regulated by developmental age in either genotype. Yet there are more than 5K genes that show a developmental difference in expression in *Dio3*+/+ mice but do show a developmental change in *Dio3*−/− mice. This number of genes is much larger than those differentially expressed between genotypes at either developmental age. This divergence suggests that for many genes, although differential expression does not achieve statistical significance at a given age, their expression trajectory during development is modified by DIO3 deficiency, an interesting possibility that requires further analyses.

In summary, we show broad differences in gene expression in the brain of fetuses with DIO3 deficiency, demonstrating that the fetal brain is sensitive to T3. The model used further indicates that *Dio3* is a critical modulator of this sensitivity and probably the main reason why other models of altered thyroid hormone status based on TH administration have shown very limited effects. Future research using this model may provide additional insights into the role of TH in early brain development.

DATA AVAILABILITY STATEMENT

RNA-sequencing fastq and processed files are publicly available at Gene Expression Omnibus (GEO) Database (Accession number: GSE172000).

ETHICS STATEMENT

The animal study was reviewed and approved by Maine Medical Center Research Institute Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MM performed the animal work, RNAScope, and real time PCR experiments, drafted the corresponding methods, results, and figures, and edited the manuscript. AH designed the study, analyzed RNA sequencing results, and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.703730/full#supplementary-material

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