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A role for zinc transporter gene *SLC39A12* in the nervous system and beyond

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Abstract

The *SLC39A12* gene encodes the zinc transporter protein ZIP12, which is expressed across many tissues and is highly abundant in the vertebrate nervous system. As a zinc transporter, ZIP12 functions to transport zinc across cellular membranes, including cellular zinc influx across the plasma membrane. Genome-wide association and exome sequencing studies have shown that brain susceptibility-weighted magnetic resonance imaging (MRI) intensity is associated with ZIP12 polymorphisms and rare mutations. ZIP12 is required for neural tube closure and embryonic development in *Xenopus tropicalis*. Frog embryos depleted of ZIP12 by antisense morpholinos develop an anterior neural tube defect and lack viability. ZIP12 is also necessary for neurite outgrowth and mitochondrial function in mouse neural cells. ZIP12 mRNA is increased in brain regions of schizophrenic patients. Outside of the nervous system, hypoxia induces ZIP12 expression in multiple mammalian species, including humans, which leads to endothelial and smooth muscle thickening in the lung and contributes towards pulmonary hypertension. Other studies have associated ZIP12 with other diseases such as cancer. Given that ZIP12 is highly expressed in the brain and that susceptibility-weighted MRI is associated with brain metal content, ZIP12 may affect neurological diseases and psychiatric illnesses such as Parkinson's disease, Alzheimer's disease, and schizophrenia. Furthermore, the induction of ZIP12 and resultant zinc uptake under pathophysiological conditions may be a critical component of disease pathology, such as in pulmonary hypertension. Drug compounds that bind metals like zinc may be able to treat diseases associated with impaired zinc homeostasis and altered ZIP12 function.

Keywords

ZIP12; brain; lung; metal; MRI; genetics

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Declarations statement

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1. The role of zinc in the nervous and other physiological systems

Zinc is a critical micronutrient involved in many body processes across the lifespan. Zinc is needed for cell division, maturation, and neuronal plasticity (Gower-Winter and Levenson, 2012). It is used as a coactivator, catalyst, and provides structural support in over 300 proteins (Takeda, 2001). Zinc is an essential mineral for development *in utero* (Wang et al., 2015) and is involved in normal brain function and development (Sandstead et al., 2000). More specifically, zinc is used as a modulator in synaptic neurotransmissions, nucleic acid metabolism, and brain tubulin growth and phosphorylation (Sandstead et al., 2000). Studies show associations between zinc deficiency in children and delayed cognitive and motor development (Black et al., 2004; Lind et al., 2004). Excess or disordered zinc influx has been linked to neurological disorders such as autism (Grabrucker et al., 2011), stroke (Sensi et al., 2009), epilepsy (Sensi et al., 2011), and schizophrenia (Scarr et al., 2016). Thus, the regulation of cellular zinc homeostasis within the nervous system is important. Outside the nervous system, zinc is also crucial for a wide range of physiological systems, including immunity, skin integrity, and reproduction (Prasad, 2008; Ogawa et al., 2016). Proteins that mediate zinc transport and homeostasis are critical components for supporting these different physiological systems.

2. The SLC39A12 gene (and ZIP12 protein) is member 12 of the solute carrier family 39 (SLC39) gene family of zinc transporters

Most of the SLC30 and SLC39 family of metal ion transporters have been shown to control intracellular zinc levels (Eide, 2004; Palmiter and Huang, 2004). In general, the SLC39 family of zinc transporters, called ZIPs or Zrt, Irt-like Proteins, are used to transport zinc into the cytoplasm, whereas the SLC30 family, called ZnT (Zn Transporters) transporters are used to transport zinc out of the cytoplasm. Members of both the ZIP and ZnT metal ion transporters contain multiple transmembrane domains. The cooperativity and concertion of zinc transport proteins provides an invaluable mechanism for regulating cellular zinc homeostasis through the body. The SLC39 family contains 14 members (Eide, 2004). ZIP12, which is encoded by the *SLC39A12* gene (OMIM 608734), contains many biochemical properties that are distinguishing hallmarks of the SLC39 gene family and associated ZIP transporters. A basic summary of the properties of the *SLC39A12* gene and ZIP12 protein is listed in Table 1. ZIP12 contains eight transmembrane domains, with one of those domains containing the amino acid sequence HEXPHEXGDFAXLLXXG (X indicates any amino acid, refers to positions with less constraint), which is hypothesized to enable zinc permeability for ZIP proteins (Taylor and Nicholson, 2003; Jenkitkasemwong et al., 2012). ZIP12 has been shown to transport zinc from the external cell media in heterologous cell models (Chowanadisai et al., 2013a; Scarr et al., 2016). In contrast, ZIP8 and ZIP14 have a glutamic acid (E) residue replacing the first histidine (EEXP, underlined) and have been shown to transport iron, manganese, or cadmium in addition to zinc (Jenkitkasemwong et al., 2012). There can be two different transcripts in ZIP12 due to the inclusion or exclusion of an in-frame exon which extends an intracellular loop with a histidine-rich region (Sensi et al., 2011). The presence of two splice variants appears to be conserved across many

vertebrates and has been experimentally detected in the brains of humans, cows, and rats, but not mice (Sensi et al., 2011). The topology of ZIP12 noted with relevant domains is displayed as Figure 1.

3. Expression pattern of ZIP12 in the vertebrate nervous system

Transcriptome data shows that ZIP12 is highly expressed in the brain in comparison to other zinc transporters in the SLC30 and SLC39 families (Chowanadisai et al., 2013a). The high expression of ZIP12 in the nervous system is also supported by expressed sequence tag (EST) data (Taylor and Nicholson, 2003; Eide, 2004). This pattern of high ZIP12 expression within the nervous system is consistent across a wide range of vertebrates, from birds to many mammals (Chowanadisai, 2014). *SLC39A12* does not appear to be present in *Drosophila* or zebrafish genomes (Chowanadisai, 2014), although there are difficulties in distinguishing between paralogs of the SLC39 family in animals that are genetically distant from humans and mice. This bioinformatics hurdle in correctly separating ZIP12 from ZIP4 and other ZIP proteins is due to high similarity of the coding sequences for *SLC39A12* and *SLC39A4* (Chowanadisai, 2014), a close member of the SLC39 family that encodes the zinc transporter ZIP4 which is mutated in acrodermatitis enteropathica (Kury et al., 2002; Wang et al., 2002). ZIP12 mRNA (Chowanadisai et al., 2013a) and protein (Zhao et al., 2015) have been reported in other tissues, and a role for ZIP12 in other non-neural tissues is discussed later in this review.

4. Importance of ZIP12 in nervous system development

ZIP12 is important for neurite outgrowth, neuronal differentiation, and microtubule polymerization and stability in mouse neuronal models (Chowanadisai et al., 2013a). Loss of ZIP12 function results in stunted neurite outgrowth and decreased cAMP response element-binding protein (CREB) activation. Although a full understanding of the mechanisms underlying the phenotypic effects seen in ZIP12-depleted cells is still relatively unknown, one piece of evidence points to impaired cell signaling and mitochondrial function (Chowanadisai et al., 2013a). Constitutive activation of CREB can restore neurite outgrowth in cells without ZIP12 or in cells exposed to media with the cell-impermeant chelator diethylentriamene pentaacetate (DTPA) (Chowanadisai et al., 2013a). CREB is known to be a transcription factor that is important for mitochondrial function (Ryu et al., 2005). Importantly, the ability for CREB to rescue neurite outgrowth in zinc-chelated media suggested that cells retained sufficient internal stores or could repurpose cellular zinc to support neurite extension without additional zinc influx from outside the cells. One explanation would be that the ZIP12 and zinc induces CREB activation and signaling during neurite outgrowth. Other researchers have demonstrated that zinc is important for cell signaling in immunoglobulin E receptor function and mast cells (Yamasaki et al., 2007).

In *Xenopus tropicalis* frog embryos, ZIP12 is necessary for neural tube closure and embryonic viability (Chowanadisai et al., 2013a). *Xenopus tropicalis* provides an approach to study rapidly study gene function in an animal embryo model with a vertebrate diploid genome (Hellsten et al., 2010). ZIP12 is first expressed at stage 13, as detected by PCR, which coincides with the start of neurulation. ZIP12 expression is observed in the anterior

neuropore at stage 17 and in the forebrain and midbrain after stage 28 following neural tube closure. When ZIP12 is depleted by antisense morpholino microinjections that target the ZIP12 translation start site or induce a frameshift in ZIP12 transcript by masking a splice site, neural tube closure at the anterior end is halted in *Xenopus tropicalis* embryos and leads to embryonic lethality. These findings support a role for ZIP12 in vertebrate nervous system development.

5. Role of ZIP12 in mitochondrial function and resistance to oxidative stress

ZIP12 supports mitochondrial function and neuronal development as evidenced by reduced neurite outgrowth, lower cellular respiration, and superoxide generation with loss of ZIP12 function (Strong et al., 2020). To investigate the role of ZIP12 in neural and mitochondrial function, Neuro-2a cells were used to model neuronal development and implement CRISPR/Cas 9-mediated genome editing and shRNA-mediated knockdown to induce the loss of ZIP12 by knockout (KO) or depletion, respectively. Studies have shown that mitochondrial motility and subcellular localization within neurons is necessary for axonal growth and development (Sheng, 2014). Similarly, lack of mitochondria at terminal ends of axons and synapses is associated with decreased axonal length (Mattson and Partin, 1999). Consistent with reduced mitochondrial function, exposure of ZIP12-deficient cells to subthreshold concentrations of inhibitors for complex I and IV of the electron transport chain (rotenone and sodium azide, respectively) significantly reduces neurite outgrowth in ZIP12-deficient cells (Strong et al., 2020). Rotenone has been shown to reduce mitochondrial movement, and increase neurite retraction and cell death (Krug et al., 2013). Further, rotenone exacerbates the production of the superoxide anion from complex I of the electron transport chain (Kudin et al., 2004), while sodium azide can induce a pro-oxidative cellular state that affects mitochondrial function (Brouillet et al., 1994). Loss of ZIP12 leads to the production of superoxide in the mitochondria and increases protein carbonylation (Strong et al., 2020). Carbonyl groups in proteins are formed as a result of oxidative damage (Suzuki et al., 2010). ZIP12 deficiency also reduces cellular respiration, which is an indicator of impaired mitochondrial function. These findings indicate that ZIP12 likely supports neurite outgrowth by increasing the availability of zinc for mitochondrial function.

In support of a role of ZIP12 in mitochondrial function, blunted neurite outgrowth in ZIP12-deficient cells is restored by overexpression of peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α) or mitochondrial superoxide dismutase (SOD2). PGC-1α is a transcriptional co-activator that binds to many nuclear respiratory transcription factors involved in mitochondrial biogenesis (Lin et al., 2005). ZIP12 can activate cAMP response element-binding protein (CREB), which is a step that is critical for neuronal differentiation (Chowanadisai et al., 2013a). CREB induces PGC-1α expression through binding cAMP response elements present in the PGC-1α promoter (St-Pierre et al., 2006; Strong et al., 2020). PGC-1α null mice show increased lesions in the brain causing impaired behavior and hyperactivity, which demonstrates a critical role for PGC-1α and mitochondrial regulation in brain function (Lin et al., 2004). Striatal neurons from PGC-1α knockout mice show reduced neurite outgrowth in histological sections and in primary

neurons cultured *in vitro* (Lin et al., 2004). Other studies note increased expression of PGC-1α when investigating the effects of mitochondria on neurite extension, highlighting its importance in neuronal development (Kambe and Miyata, 2012).

The neuroprotective property of PGC-1α in ZIP12-deficient cells can be tied to mitochondrial biogenesis and positive regulation of antioxidant defense enzymes (Strong et al., 2020). Oxidative stressors like hydrogen peroxide induces the expression of PGC-1α, which in turn promotes a mitochondrial metabolic gene program that can detoxify reactive oxygen species (St-Pierre et al., 2006). St-Pierre et al. (St-Pierre et al., 2006) found that PGC-1α enhances expression of many antioxidant enzymes including superoxide dismutases SOD1 and SOD2. Overexpression of PGC-1α in both human SH-SY5Y neuroblastoma cells and primary mouse striatal neurons confers enhanced cell survival and resilience to compounds such as paraquat and hydrogen peroxide that induce oxidative stress. Mitochondrial superoxide dismutase SOD2, a downstream target of PGC-1α (St-Pierre et al., 2006), significantly rescues neurite outgrowth in ZIP12-deficient cells. In contrast, cytosolic SOD1 failed to restore neurite outgrowth in ZIP12-deficient cells, which may reflect the localization of SOD2 within the mitochondria. Interestingly, PGC-1α expression is reduced in neurodegenerative diseases such as Huntington disease and Parkinson's disease (Cui et al., 2006). PGC-1α knockout mice show increased sensitivity to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a complex I inhibitor, which results in increased oxidative stress and cell death in the substantia nigra (St-Pierre et al., 2006). Transfection of PGC-1α or SOD2 can restore neurite outgrowth in ZIP12-depleted and ZIP12-deleted cells (Strong et al., 2020). Thus, ZIP12 and zinc likely has a role in the regulation of mitochondrial function, a process which is commonly disturbed in many neurodegenerative diseases (Strong et al., 2020). Furthermore, the application of antioxidants such as α-tocopherol, MitoQ, and MitoTEMPO is able to also restore neurite outgrowth in ZIP12 KO cells (Strong et al., 2020), further emphasizing the role of oxidative stress in neural dysfunction due to lack of ZIP12.

6. UK Biobank as a resource to study role of ZIP12 in human brain function and structure

The establishment of new biological and health databases in conjunction with genomic sequencing and polymorphism detection allows for investigation into novel genetic components and their associated biological effects. This strategy of mining health and genetics data was used to explore links between ZIP12 polymorphisms and susceptibility weighted magnetic resonance imaging (swMRI) phenotypes in the human brain. The UK Biobank data repository contains information from 500,000 participants at ages 40–69 years old during recruitment and followed for up to 30 years (Bycroft et al., 2018). A wide range of phenotypic data, including dietary assessment, cognitive performance, health history, and biomarker data like blood, urine, and saliva is available to researchers for analysis (Bycroft et al., 2018). Dietary recall is recorded through the Oxford WebQ questionnaire (Galante et al., 2016). Cognitive function is assessed through a touchscreen computer and shows longitudinal stability (Lyall et al., 2016) and reliability with other in-person, reference measures of cognition (Fawns-Ritchie and Deary, 2020). In addition, brain imaging data for

100,000 subjects was collected by MRI and automated post-collection image processing (Miller et al., 2016). Genomic DNA was also collected, in order to enable genetic links to the phenotypic data (Bycroft et al., 2018). Whole genome arrays cover over 800,000 markers with close to 100 million polymorphisms after imputation (Bycroft et al., 2018). Exome sequencing enables detection of rare coding variants (Cirulli et al., 2020).

Secondary analyses of the summary statistics dataset by Elliott et al. (Elliott et al., 2018) uncovered associations between coding variants of ZIP12 and brain swMRI intensity in the caudate, the putamen, and the pallidum, which are 3 regions of the basal ganglia (Strong et al., 2020). Associations between polymorphisms in SLC39A12 and brain MRI phenotypes were first discovered through a genome-wide association study (GWAS) by Elliott et al. (Elliott et al., 2018). In subjects from the UK Biobank, subjects were imaged under a wide array of different MRI conditions, including structural T1 and T2 imaging, diffusion MRI, and resting and task-oriented functional MRI, and genotyped with a genome-wide SNP array (Elliott et al., 2018). In all 3 brain regions assessed (caudate, putamen, pallidum), the highest marker is one of two intergenic, non-coding polymorphisms, rs10430577 or rs10430578. These lead polymorphisms are also associated with differences in T1 FAST imaging, which is a measure commonly related to gray matter volume (Elliott et al., 2018). Conditional and joint association analyses showed that in addition to rs10430577 and rs10430578, there were additional independent association signals associated with swMRI in the putamen and pallidum. These association signals (rs10827902, rs72784718) are in high linkage disequilibrium with two coding polymorphisms (rs10764176, rs72778328), and these ZIP12 coding polymorphisms reduce zinc transport activity relative to the reference version (Strong et al., 2020). In addition, rare heterozygous variants in ZIP12, which are present in 36 of 9965 total individuals and predicted to be deleterious, are associated with altered brain MRI phenotypes in a similar fashion to the coding ZIP12 polymorphisms with reduced zinc transport activity (Cirulli et al., 2020; Strong et al., 2020). These studies in humans complement our previous findings in cellular and developmental models (Chowanadisai et al., 2013a) showing that ZIP12 can affect the human brain in a way that is detectable by MRI (Strong et al., 2020).

7. A possible role for ZIP12 in protection from neurodegeneration

Numerous studies have linked neurodegenerative disorders to mitochondrial dysfunction. For example, mitochondrial function is impaired in dopaminergic neuronal cultures *in vitro* and post-mortem brain tissue from patients with Parkinson's disease (Devi et al., 2008). Neuronal cells isolated from mice with mutant alpha-synuclein associated with Parkinson's disease show increased cell death in the neocortex, brain stem, and spinal cord along with degenerating mitochondria (Martin et al., 2006). Inhibition of complex I with rotenone results in the loss of dopaminergic neurons and motor deficits, which are hallmarks of Parkinson's disease (Betarbet et al., 2000). Post-mortem studies on Alzheimer diseased brains show declined activity in complex IV (Selvatici et al., 2009). Transgenic Alzheimer's disease mice have increased lipid peroxidation and hydrogen peroxide production (Yao et al., 2009), which is consistent with induced oxidative stress from mitochondrial dysfunction. Rats continually exposed to sodium azide through osmotic pumps have impaired spatial memory (Bennett and Rose, 1992), which is similar to symptoms reported in Alzheimer's

disease. Because the loss of ZIP12 results in increased sensitivity to rotenone and sodium azide (Strong et al., 2020), researchers explored whether transfection of mutated variants of tau and alpha-synuclein would further impair neurite outgrowth in ZIP12-deficient cells. Tau protein mutant P301L is found in patients with frontotemporal dementia, a disease that is similar to Alzheimer's disease (Hutton et al., 1998). Cells without ZIP12 and expressing tau protein mutant P301L have significant deficits in neurite outgrowth, leading to roughly 80 percent fewer cells with neurites relative to ZIP12 KO cells transfected with wildtype tau protein. Similar impacts on neurite extension were found for cells expressing alpha-synuclein protein mutant A53T but only for ZIP12 KO cells and not cells with ZIP12-depletion by shRNA. These results are consistent with an added burden of mitochondrial dysfunction and oxidative stress in cells already compromised by ZIP12 deficiency. More studies are needed to determine if our observations about the loss or reduced function of ZIP12 and associations with mitochondrial dysfunction and sensitivity to neurodegeneration have clinical relevance.

Although differences in brain structure observable by MRI are commonly seen in neurological disorders (Draganski et al., 2013), additional studies will be needed to determine if ZIP12 is important for resilience from brain aging and neurodegenerative diseases. Considering that UK Biobank will follow human subjects in a prospective fashion and a wide range of phenotyping data is being recorded, including cognitive data, it may be possible to determine whether ZIP12 polymorphisms or rare variants affect human brain aging. Furthermore, other metal metabolism genes in addition to *SLC39A12* may affect brain structure and produce other neural phenotypes. Elliott et al. (Elliott et al., 2018) have already identified iron metabolism genes such as TF (transferrin), HFE (high Fe, iron), FTH1 (ferritin, heavy-chain), and COASY (coenzyme A synthase) as being associated with brain swMRI. In addition, ZIP8 and ZIP14 are also linked to swMRI in the basal ganglia (Elliott et al., 2018), though these two transporters exhibit broader selectivity for metals than ZIP12 (Jenkitkasemwong et al., 2012). One limitation of the UK Biobank is that it is not representative of all ethnic and racial groups. The establishment of new resources such as the National Institutes of Health All of Us research initiative (All of Us Research Program et al., 2019) may introduce more diversity to health and genetic repositories. In a similar fashion to how there is positive and directional selection for a coding variant of ZIP4 in the sub-Saharan African population (Engelken et al., 2014), missense ZIP12 variant rs11011935 is most prevalent in the African population with an allele frequency of 0.238, whereas the frequency is below 0.001 in European and Asian populations (Zhang et al., 2015). Given these observations in cellular models, it is possible that rare mutations or possibly common polymorphisms in SLC39A12 may increase susceptibility to neurodegenerative diseases.

8. SLC39A12 and autism

Currently, it is unclear whether genetic variability in *SLC39A12* contributes towards autism risk. In one study assessing copy number variations in 104 Han Chinese autistic patients, a heterozygous deletion in *SLC39A12* was detected (Gazzellone et al., 2014). In another study with 2,377 families affected by autism, a premature stop codon was detected in one copy of *SLC39A12* for one proband (Krumm et al., 2015). Autistic patients are more likely to have truncating single nucleotide variants compared to unaffected siblings (Krumm et al., 2015),

although a wide array of genes are affected which reflects the complex polygenic nature of the disorder. ZIP12 polymorphisms and rare variants are associated with swMRI in the basal ganglia and total putamen gray volume (Chowanadisai et al., 2013a), and differences in caudate and putamen volume have been detected in autistic patients (Hollander et al., 2005). Given that zinc is important for brain development (Grabrucker et al., 2011) and the requirement for ZIP12 in neurulation and early neurodevelopment (Chowanadisai et al., 2013a), an impact of ZIP12 in autism risk would be plausible (Chowanadisai et al., 2013b).

9. ZIP12 in schizophrenia

Bly detected a possible connection between ZIP12 and schizophrenia by sequencing 102 patients with schizophrenia from three different collections and 111 control subjects without schizophrenia (Bly, 2006). The frequency of homozygotes for a non-coding ZIP12 polymorphism (rs10764176, S36G) was nominally elevated in the schizophrenic group (11.3%) compared to controls (5.4%). However, this finding did not meet the statistical bar for significance given the limited sample size (Bly, 2006). Subsequent GWAS on schizophrenia with significantly larger sample pools and using high-density SNP arrays have failed to identify SLC39A12 as an associated locus (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Lam et al., 2019), but a significant association between schizophrenia and an adjacent gene CACNB2 has been noted (Schizophrenia Working Group of the Psychiatric Genomics, 2014; Lam et al., 2019).

Schizophrenia is associated with differences in ZIP12 mRNA abundance and suggest that alterations in brain zinc metabolism affect psychiatric illnesses (Scarr et al., 2016). Scarr et al. (Scarr et al., 2016) used genome-wide microarrays to assess transcriptome differences in cortical regions in subjects with schizophrenia. In this study, brain tissue was collected from the left hemispheres of the frontal lobe, superior frontal gyrus, and inferior frontal gyrus, which correspond to Brodmann's areas (BA) 9, 8, and 44, respectively. Gene expression was compared to controls with no history of psychiatric illness and subjects affected by different mood disorders, either major depressive disorder or bipolar disorder. Among the schizophrenia group, the subjects were further sub-divided into subjects with or without the muscarinic receptor deficit schizophrenia (MRDS) subtype, which was characterized by a marked decrease in muscarinic M1 receptor mRNA in the cortex (Scarr et al., 2018).

In the Scarr study, ZIP12 was found to be the most differentially expressed gene in BA 9 and 44 (Scarr et al., 2016). Both splice variants of ZIP12 were more highly expressed in schizophrenic subjects in all 3 brain regions examined (frontal lobe, superior frontal gyrus, and inferior frontal gyrus) when compared to control subjects without mental illness. Accounting for MRDS subtype, ZIP12 variant 1 (long splice variant with additional exon inclusion) was higher in BA 8 and BA 9 of schizophrenics with the MRDS subtype but not in schizophrenic subjects from the non-MRDS group. For variant 2 (short variant due to exon exclusion), ZIP12 mRNA content was higher in BA 9 for MRDS subjects but not for non-MRDS patients. For BA 8, ZIP12 transcript variant 2 was higher in both MRDS and non-MRDS groups. For BA 44, both ZIP12 variants were higher regardless of MRDS status. In summation, the mRNA abundance of only one of the two ZIP12 variants was influenced by MRDS subtype. Differences in MRDS status was found to be associated with differential

expression of 65 other genes (including ZIP12), and it is proposed that these gene pathways lie upstream of the muscarinic M1 receptor (Scarr et al., 2018). It is possible that these upstream pathways may affect or interact with ZIP12 expression and also affect one transcript more than the other. The study also demonstrated that both splice variants are able to transport zinc, implying that the histidine-rich internal loop that distinguishes these variants is not directly involved in zinc transport (Chowanadisai, 2014).

Because it is possible that the difference in ZIP12 mRNA transcripts are due to medications use to treat schizophrenia, ZIP12 mRNA expression was measured in rats injected with haloperidol, chlorpromazine, or thioridazine. No differences in ZIP12 expression were observed in the cortex of the drug-injected rats when compared to vehicle-injected controls. This data supports the notion that the observed differences in ZIP12 expression in the brain of schizophrenics are due to the biological effects of mental illness prior to treatment rather than an induced response to medication. In summary, this study showed that ZIP12 expression is associated with schizophrenic neuropathology in cortical regions of BA 8, 9, and 44, suggesting ZIP12 and its ability to transport zinc may play a key role in schizophrenia.

10. Role of ZIP12 in lung pathophysiology

ZIP12 is involved in pulmonary vascular remodeling, as demonstrated in a study identifying genetic differences between Fisher 344 (F344) rats, which are resistant to hypoxia-induced pulmonary hypertension, and susceptible Wistar Kyoto (WKY) rats (Zhao et al., 2015). Resistant F344 rats crossed with non-resistant WKY rats produce subcongenic strains for quantitative trait loci (QTL) analysis. The QTL was narrowed down to approximately 65 genes on rat chromosome 17, which corresponds to human chromosome 10. Whole genome sequencing of parent strains F344 and WKY and analysis of genes of interest narrowed the candidates to seven possible genes within the QTL, including *SLC39A12*. A ZIP12 frameshift mutation in F344 rats truncates 20 percent of the protein at the C-terminus and lacks the putative metal-transporting motif, which should impair cellular zinc uptake. Knockout of the ZIP12 protein using zinc finger nucleases renders WKY rats resistant to hypoxia-induced pulmonary hypertension, confirming that *SLC39A12* is the causative gene.

ZIP12 is ordinarily expressed at low or undetectable levels in many tissues outside of the nervous system, but hypoxia can induce ZIP12 expression (Zhao et al., 2015). In environments with normal oxygen content, ZIP12 mRNA expression is minimal in WKY rats sensitive to pulmonary hypertension. However, in hypoxic environments a marked increase in ZIP12 mRNA expression is apparent in multiple cell types related to pulmonary vascular remodeling, including vascular smooth muscle, endothelial, and interstitial cells. In contrast, ZIP12 is not detected by immunohistochemistry in F344 rats exposed to chronic hypoxic environments. Furthermore, other species, specifically cattle and humans, show increased expression of the ZIP12 protein when housed in hypoxic environments, which demonstrated that the response of ZIP12 to hypoxia was consistent across mammals. Human pulmonary vascular smooth muscle cells cultured in 2% oxygen also exhibit increased ZIP12 mRNA and protein levels. The induction of ZIP12 due to hypoxia can be traced to a hypoxia response element (HRE) within a *SLC39A12* intron with the ability to bind hypoxia

inducible factors HIF-1α and HIF-2α. Furthermore, under hypoxia, pulmonary artery smooth muscle cells exhibit increased zinc content and cell proliferation, which is not present when ZIP12 is depleted with short interfering RNA. The proliferation of vascular smooth muscles is a hallmark of pulmonary hypertension due to hyperplasia and hypertrophy which thickens the pulmonary vasculature. Chronically iron-deficient rats have increased ZIP12 expression caused by impaired oxygen transport (Zhao et al., 2015), which may point to links between iron and zinc metabolism in the brain or other tissues like lung involving ZIP12.

In a study by Abdo and colleagues using human vascular endothelial and smooth muscle cells, ZIP12 expression increased by exposure to cell-permeant zinc chelator N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) (Abdo et al., 2020). These findings are consistent with our findings showing an increase in ZIP12 protein expression in Neuro-2a cells following zinc chelation (Chowanadisai et al., 2013a). Abdo et al. also found that ZIP12 is enriched at the lamellipodia of cells (Abdo et al., 2020). Intriguingly, in neurons, neurites extend from segmented lamellipodia (Dehmelt et al., 2003), and neurite outgrowth is disrupted in ZIP12-deficient cells (Chowanadisai et al., 2013a).

Given that different strains of rats show variability in pulmonary vascular responses and smooth muscle thickening due to genetic differences in ZIP12 (Zhao et al., 2015), it is possible that such genetic variability in ZIP12 may alter human susceptibility to pulmonary remodeling from hypoxia. Perhaps polymorphisms or mutations in ZIP12 may confer susceptibility or resilience to diseases outside of the nervous system, given the tight control of zinc and consequences for mis-regulation of zinc homeostasis.

11. Additional roles for ZIP12 outside of the nervous system

ZIP12 may have roles outside of the nervous system in either a physiological or pathophysiological context. Although transcriptome data support a role for ZIP12 in the brain, other studies have reported associations or roles for ZIP12 in other tissues. For example, ZIP12 has been shown to be important in lung pathophysiology in response to hypoxia. It is likely that ZIP12 may be associated with other diseases as well. Although transcriptome data supports a role for ZIP12 in the brain, it is possible that ZIP12 may have a biological impact in other peripheral tissues as part of its normal function or in disease states. In mice, the ZIP12 mRNA transcript is highest in the brain, followed by lung, and can be detected in heart, kidney, liver, muscle, small intestine, colon, and thymus (Strong et al., 2020). ZIP12 mRNA was not present in the mouse pancreas (Strong et al., 2020). ZIP12 protein has been detected in the mouse brain (Strong et al., 2020) and lung (Zhao et al., 2015). It is possible that ZIP12 protein may be present and important in other tissues and be important, although the level of expression is likely to be lower than in the nervous system (Strong et al., 2020).

ZIP12 has been associated with biological and health effects in different vertebrates. In broiler male chicks, ZIP12 mRNA expression decreases in response to an oral challenge with *Salmonella typhimurium* and parallels changes with expression of other ZIP transporters, such as ZIP5, ZIP10, ZIP11, ZIP13, and ZIP14 (Wu et al., 2020). These

decreases in mRNA expression among multiple ZIP transporters may be a response by the duodenum and lead to decreased zinc uptake and subsequent hypozincemia (Wu et al., 2020). Others have reported that ZIP12 mRNA and protein expression is increased in lung and liver of chickens following induction of ascites syndrome by intravenous cellulose microparticle injection (Cui et al., 2019). In zinc-deficient T-cells, the addition of zinc to the media stimulates expression of ZIP10 and ZIP12, which has been proposed to promote cytokine production by the immune system as an inflammatory response (Daaboul et al., 2012). Using quantitative trait loci mapping in 2 different bovine strains, it was found that ZIP12 may be a candidate for impacting female fertility in Chinese and Nordic Holsteins (Liu et al., 2017). However, it should be noted that *CACNB2*, a calcium channel gene located adjacent to *SLC39A12* (Chowanadisai, 2014), was also associated with fertility and controls the secretion of follicle stimulating hormone (FSH) in cows (Sugimoto et al., 2013). ZIP12 mRNA expression is higher in murine oocytes compared to cumulus cells, which may point to a role for zinc transport by ZIP12 in reproduction and fertility (Lisle et al., 2013). A genome-wide association study in horses has linked an intronic polymorphism in ZIP12 to endurance racing performance in Arabian horses, as determined by race finishing status (Ricard et al., 2017). Although it is unclear how ZIP12 may be affecting endurance, genes linked to nervous system function can promote exercise endurance in mice (Yaghoob Nezhad et al., 2019).

One study has suggested that ZIP12 may have a role in glucose metabolism. Ge et al. (Ge et al., 2018) showed that two single nucleotide polymorphisms in *SLC39A12*, rs2497753 and rs9418383, are associated with elevated fasting plasma glucose in a GWAS involving 511 Chinese adults. Additionally, both SNPs are associated with altered glycosylation patterns with immunoglobulin G (IgG), which may be a biomarker for persistently elevated plasma glucose (Ge et al., 2018). However, more studies are needed to confirm these findings. To date, *SLC39A12* has not been identified as a diabetes risk gene in GWAS, even with trans-ethnic populations and subject populations exceeding 100,000 included in the meta-analysis (Replication et al., 2014). We previously showed that ZIP12 mRNA is undetectable in human and mouse pancreas (Chowanadisai et al., 2013a), which is supported by another study examining ZIP12 expression in pancreatic beta-cells (Lawson et al., 2017). If ZIP12 is not present in the pancreas, ZIP12 polymorphisms likely impact fasting plasma glucose through other tissues. One question would be whether ZIP12 in the nervous system can affect systemic glucose metabolism or peripheral insulin sensitivity, or whether ZIP12 outside of the nervous system is responsible.

Although we were previously unable to find ZIP12 protein expression in peripheral tissues outside of the nervous system (Chowanadisai et al., 2013a), the ability of some of these studies to detect ZIP12 may be due to the induction of various pathological conditions, which may increase ZIP12 protein to the point of detection. As an example of how ZIP12 can be difficult to identify outside of the central nervous system, ZIP12 mRNA was undetectable in peripheral blood mononuclear cells of most human subjects tested by quantitative RT-PCR (Wex et al., 2014). Given that ZIP12 has been implicated in diseases such as pulmonary hypertension (Zhao et al., 2015), the role of ZIP12 outside the nervous system in disease states may be of future research interest.

Altered expression and mutations in ZIP12 have been reported in different cancers, which is consistent with known links between zinc and cancer (Ziliotto et al., 2018). Mutations in ZIP12 have been detected in esophageal adenocarcinoma by whole exome sequencing (Dulak et al., 2013). In 145 patients with esophageal adenocarcinoma, 12 patients had ZIP12 missense mutations in tumors that were negative for microsatellite instability (Dulak et al., 2013). In esophageal adenocarcinoma tumors from 8 patients, two coding mutations in ZIP12 were present in different patients (Murugaesu et al., 2015). In a region of one tumor, mutation A44E in ZIP12 was present in all cancerous cells, although this mutation was not detected in other tumor regions from the same patient (Murugaesu et al., 2015). In another tumor, ZIP12 mutation T37I and a *TP53* splice site mutation were present in all regions, which demonstrated that both of these mutations were clonal in origin (Murugaesu et al., 2015). Aberrant ZIP12 expression has also been reported in different cancers. For example, in one study, ZIP12 mRNA was overexpressed in many non-small cell lung cancer biopsied tissues from at least half of tested patients (Huang et al., 2016). In contrast, ZIP12 protein abundance was significantly lower in luminal breast cancer line T47D and basal breast cancer line MDA-MB-231 relative to non-malignant mammary cell line MCF10A (Chandler et al., 2016). Given that ZIP4 overexpression has been detected in cancerous tissues (Li et al., 2007) and ZIP6 (LIV-1) can activate epithelial-mesenchymal transition (Hogstrand et al., 2013), a potential role for closely related zinc transporter ZIP12 in cancer is worth investigating.

12. Conclusion

Consistent with the expression profile of ZIP12, there appears to be a role for ZIP12 in the nervous system that influence mitochondrial function. Genome-wide association and rare variant studies show that genetic variability at *SLC39A12* can impact susceptibility-weighted brain MRI intensity, although more research is needed to demonstrate what physiological changes are causing these alterations detected by brain MRI and if they are associated with the mitochondria. In addition, ZIP12 may affect brain zinc homeostasis and be disrupted in schizophrenia or autism. Additional research shows that ZIP12 has significant relevance outside the nervous system, although in some of these cases, they may be due to pathophysiological conditions such as hypoxia in pulmonary systems or cancer. These studies to date, which are summarized in Tables 2 and 3, point to an emerging role for ZIP12 and its contribution to cellular zinc homeostasis. Importantly, pharmaceutical compounds that bind metals, such as zinc ionophores (Cherny et al., 2001), may have therapeutic value in diseases associated with impaired ZIP12 function.

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Abbreviations list

BA	Brodmann's areas
COASY	coenzyme A synthase
CREB	cAMP response element-binding protein
DTPA	diethylentriamene pentaacetate
EST	expressed sequence tag
F344	Fisher 344
FSH	follicle stimulating hormone
FTH1	ferritin, heavy-chain
GWAS	genome-wide association study
HFE	high Fe, iron
HRE	hypoxia response element
KO	knockout
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRDS	muscarinic receptor deficit schizophrenia subtype
MRI	magnetic resonance imaging
PGC-1α	peroxisome proliferator-activated receptor-gamma coactivator-1alpha
QTL	quantitative trait loci
shRNA	short hairpin RNA
SOD1	superoxide dismutase 1
SOD2	superoxide dismutase 2
SNP	single nucleotide polymorphism
swMRI	susceptibility weighted magnetic resonance imaging
TPEN	N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine
TF	transferrin
WKY	Wistar Kyoto
ZIPs	Zrt, Irt-like Proteins

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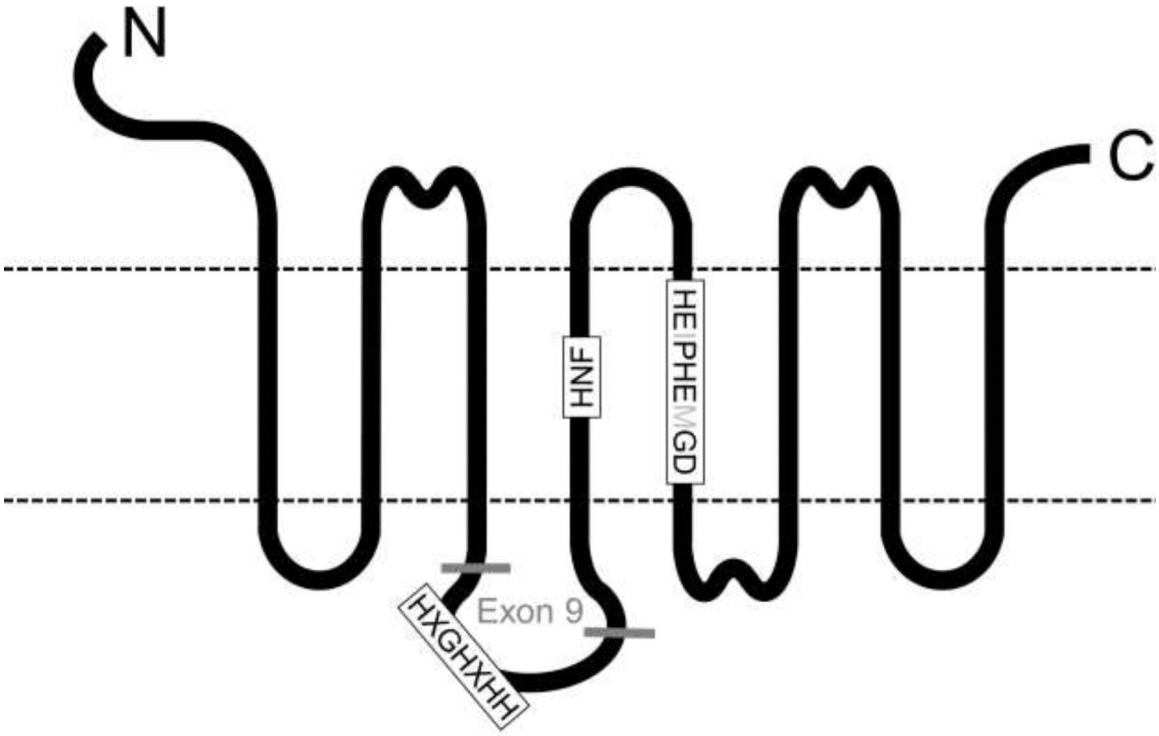

Figure 1. Topology of human ZIP12.

N and C indicate N-terminus and C-terminus, respectively. Dashed lines indicate transmembrane domains. Inclusion of exon 9, marked in gray, results in a splice variant with a longer cytoplasmic loop containing a histidine-rich region (motif HXGHXHH, X indicates amino acids not conserved across mammals). Motifs HNF and HEIPHEMGD in transmembrane regions 4 and 5 are fully conserved across different vertebrates and largely conserved across ZIP proteins. Amino acids in light gray are partially conserved between human ZIP4.

Table 1:

Properties of SLC39A12 gene and ZIP12 protein

	Human (*Homo sapien*)	**Mouse (*Mus musculus*)**
Gene symbol	*SLC39A12*	*Slc39a12*
Protein symbol	ZIP12	Zip12
Chromosome	10	2
NCBI Gene ID	221074	277468
NCBI mRNA ID	NM_001145195, NM_152725	NP_001099594
NCBI protein ID	NP_001138667, NP_689938	NP_001012305
Uniprot Entry ID	Q504Y0	Q5FWH7
Ensembl Gene ID	ENSG00000148482	ENSMUSG00000036949
Exons	13	13
Protein length (amino acids)	691 aa, 654 aa	689 aa
Predicted protein mass (kD)	72.9 kD, 76.7 kD	76.2 kD

Predicted protein mass (kD or kilodaltons) is calculated from amino acid (aa) sequence. Two additional splice variants for human ZIP12 (NM_001282733, NM_001282734) are present in the NCBI database; however to our knowledge, these variants have not been confirmed experimentally.

Table 2.

Mutations or polymorphisms in or near SLC39A12 associated with diseases or phenotypes

Disease or phenotype	Animal	Mutation or polymorphism	Citations
brain susceptibility-weighted MRI	human	rs10430577, rs10430578 (intergenic)	Elliott et al., 2018
brain T1 FAST MRI	human	rs10430577, rs10430578 (intergenic)	Elliott et al., 2018
brain susceptibility-weighted MRI	human	T72P, V111G, D166G, C347X (premature stop codon), A416T, K463I	Cirulli et al., 2020
brain susceptibility-weighted MRI	human	rs10764176 (S36G, in LD with rs10827902), rs72778328 (Q342R, in LD with rs72784718)	Strong et al., 2020; Elliott et al., 2018
schizophrenia	human	rs10764176 (S36G)	Bly, 2006
autism	human	73 kb deletion (missing SLC39A12 exons 1–12)	Gazzellone et al., 2014
autism	human	Y369X (premature stop codon)	Krumm et al., 2015
esophageal adenocarcinoma	human	R4W, T24S, G80R, Q181H, H272R, L316P, Q355H, L418P, E515D, S526I, L545M, L597P	Dulak et al., 2013
esophageal adenocarcinoma	human	T37I, A44E	Murugaesu et al., 2015
fasting plasma glucose, IgG glycoyslation	human	rs2497753, rs9418383 (intron)	Ge et al., 2018
hypoxia-induced pulmonary hypertension	rat	G544EfsX11 (1 bp deletion and frameshift); mutation is present in Fisher 344 strain and absent in Wistar Kyoto strain	Zhao et al., 2015
female fertility	cow	rs41609782, rs109983109	Liu et al. 2017
endurance (racing performance)	horse	29:1758059, 29:1758099 (intron)	Ricard et al., 2017

For polymorphisms, rsIDs for single-nucleotide polymorphisms (SNPs) are listed as reported. Missense, premature stop, and frameshift mutations within coding regions (exons) are listed according to reference amino acid position (and listed in parentheses after rsID). LD indicates coding polymorphisms in linkage disequilbrium with lead SNPs from published GWAS.

Locations within introns or between genes (intergenic) are listed as reported. Chromosome and genomic coordinates for horse are according to *Equus caballus* reference genome (EquCab 2.0).

Table 3.

Summary of ZIP12 in the nervous system and in other tissues or diseases

Tissue/organ, physiological system, or disease	**Citations**
Brain and nervous system	
Expression pattern: ZIP12 expression is highest in the brain and nervous system across vertebrates.	Chowanadisai et al., 2013a; Chowanadisai, 2014
Neural cell development: ZIP12 is necessary for neurite extension mouse Neuro-2a cells and primary mouse neurons. ZIP12 is also necessary for cellular respiration and mitochondrial function. ZIP12-deleted cells have reduced cellular respiration and are sensitive to rotenone and sodium azide. ZIP12-deleted cells have increased superoxide generation and higher oxidative damage. Exposing ZIP12-deleted cells to antioxidant chemicals can restore neurite length.	Chowanadisai et al., 2013a; Strong et al., 2020
Embryonic development: ZIP12 is expressed in the forebrain, midbrain, and eye of *Xenopus tropicalis* during nervous system development. ZIP12 is also expressed at the anterior neuropore during neural tube closure. Embryos injected with antisense morpholinos targeting ZIP12 have impaired neurulation, lack eyes, and premature lethality.	Chowanadisai et al., 2013a
Human brain MRI patterns: ZIP12 polymorphisms and rare mutations are linked to altered swMRI intensity and T1 FAST MRI in the human brain. Polymorphisms rs10430577 and rs10430578 are the lead SNPs most associated with swMRI intensity in the caudate, putamen, and pallidum and T1 FAST MRI. Missense ZIP12 mutations (rs10764176, rs72778328) associated with swMRI have reduced zinc transport activity.	Elliott et al., 2018; Cirulli et al., 2020; Strong et al., 2020
Schizophrenia: A non-coding polymorphism in ZIP12 has been reported in in patients with schizophrenia. ZIP12 mRNA is higher in frontal lobe, superior frontal gyrus, and inferior frontal gyrus of brains from schizophrenic subjects.	Bly, 2006; Scarr et al., 2016
Autism: One person (Han Chinese) with autism had a heterozygous deletion in *SLC39A12*. In a separate study, a premature stop codon was detected in one copy of *SLC39A12* in a person with autism.	Gazzellone et al., 2014; Krumm et al., 2015
Other tissues and physiological systems or diseases	
Lung and pulmonary hypertension: Fisher 344 (F344) rats are resistant to hypoxia-induced pulmonary hypertension, and Wistar Kyoto (WKY) rats are susceptible. A ZIP12 frameshift mutation present in F344 rats has reduced cellular zinc uptake activity. ZIP12 knockout rats also are resistant to hypoxia-induced pulmonary hypertension and vascular remodeling. Rats, cattle, and humans have increased ZIP12 protein when housed in hypoxic environments, which is due to a hypoxia response element (HRE) is present within a *SLC39A12* intron. ZIP12 expression increased in human vascular endothelial and smooth muscle cells after exposure to zinc chelator TPEN.	Zhao et al., 2015; Abdo et al., 2020
Cancer: ZIP12 missense mutations were found in two studies on esophageal adenocarcinoma. ZIP12 mRNA was elevated in non-small cell lung cancer biopsies. ZIP12 protein abundance was lower in the breast cancer lines relative to a non-malignant mammary cell line.	Dulak et al., 2013; Murugaesu et al., 2015; Huang et al., 2016; Chandler et al., 2016
Ascites syndrome: ZIP12 mRNA and protein increased in lung and liver of chickens after ascites syndrome by intravenous cellulose microparticle injection.	Cui et al., 2019
Immune system: The restoration of zinc to zinc-deficient T-cells induces ZIP12 expression, which may promote cytokine production by the immune system. In broiler male chicks, ZIP12 mRNA expression in the duodenum, a region of the small intestine, decreases in response to an oral challenge with *Salmonella*.	Daaboul et al., 2012; Wu et al., 2020
Fertility and reproduction: SLC39A12 (ZIP12) may be a candidate gene that affects fertility in female Chinese and Nordic Holstein cows. ZIP12 mRNA is more abundant in mouse oocytes compared to cumulus cells.	Liu et al., 2017; Lisle et al., 2013
Endurance: An intronic polymorphism in *SLC39A12* is linked to endurance racing performance in Arabian horses.	Ricard et al., 2017
Glucose metabolism: Two SNPs in SLC39A12, rs2497753 and rs9418383, were reported to be associated with elevated fasting plasma glucose and IgG glycosylation in 511 Chinese adults.	Ge et al., 2018