Loss of slc39a14 causes simultaneous manganese deficiency and hypersensitivity in zebrafish

Karin Tuschl1-3, Richard J White4,5, Leonardo E Valdivia1,6, Stephanie Niklaus7, Isaac H Bianco6, Ian M Sealy4,5, Stephen CF Neuhaus8, Corinne Houart2, Stephen W Wilson1, Elisabeth M Busch-Nentwich4,5

1 Department of Cell and Developmental Biology, University College London, Gower Street, WC1E 6BT, UK
2 Department of Developmental Neurobiology and MRC Centre for Neurodevelopmental Disorders, IoPPN, Kings College London, New Hunt’s House, Guy’s Campus, London, SE1 1UL, UK
3 UCL GOS Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK
4 Wellcome Sanger Institute, Wellcome Genome Campus, CB10 1SA, UK
5 Cambridge Institute of Therapeutic Immunology & Infectious Disease (CITIID), Jeffrey Cheah Biomedical Centre, University of Cambridge, Puddicombe Way, Cambridge, CB2 0AW
6 Center for Integrative Biology, Facultad de Ciencias, Universidad Mayor, Santiago, Chile
7 Institute of Molecular Life Sciences, University of Zurich, Winterthurerstrasse 190, 8057, Zurich, Switzerland.
8 Department of Neuroscience, Physiology & Pharmacology, University College London, Gower Street, WC1E 6BT, UK

Corresponding authors:
k.tuschl@ucl.ac.uk, https://orcid.org/0000-0001-8599-8516
emb81@cam.ac.uk, https://orcid.org/0000-0001-6450-744X

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Abstract

Mutations in SLC39A14, a manganese uptake transporter, lead to a neurodegenerative disorder characterised by accumulation of manganese in the brain and rapidly progressive dystonia-parkinsonism (Hypermanganesemia with Dystonia 2, HMNDYT2). Similar to the human phenotype, zebrafish slc39a14U801-/- mutants show prominent brain manganese accumulation and abnormal locomotor behaviour. In order to identify novel potential targets of manganese neurotoxicity, we performed transcriptome analysis of individual homozygous mutant and sibling slc39a14U801 zebrafish at five days post fertilisation unexposed and exposed to MnCl2. Anatomical gene enrichment analysis confirmed that differentially expressed genes map to the central nervous system and eye. Biological interpretation of differentially expressed genes suggests that calcium dyshomeostasis, activation of the unfolded protein response, oxidative stress, mitochondrial dysfunction, lysosomal disruption, apoptosis and autophagy, and interference with proteostasis are key events in manganese neurotoxicity. Differential expression of visual phototransduction genes also predicted visual dysfunction in mutant larvae which was confirmed by the absence of visual background
adaption and a diminished optokinetic reflex. Surprisingly, we found a group of differentially expressed genes in mutant larvae that normalised upon MnCl₂ treatment suggesting that, in addition to neurotoxicity, manganese deficiency is present either subcellularly or in specific cells or tissues. This may have important implications for treatment as manganese chelation may aggravate neurological symptoms. Our analyses show that slc39a14<sup>U801</sup> mutant zebrafish present a powerful model to study the cellular and molecular mechanisms underlying disrupted manganese homeostasis.

**Significance statement**

Manganese neurotoxicity leading to progressive dystonia-parkinsonism is a characteristic feature of Hypermanganesemia with dystonia 2 (HMNDYT2) caused by mutations in SLC39A14, a manganese uptake transporter. Transcriptional profiling in slc39a14<sup>U801</sup> loss-of-function zebrafish suggests that, in addition to manganese neurotoxicity, subcellular or cell type specific manganese deficiency contributes to the disease phenotype. Both manganese overload and deficiency appear to be associated with Ca²⁺ dyshomeostasis. We further demonstrate that activation of the unfolded protein response, oxidative stress, mitochondrial dysfunction, apoptosis and autophagy, and disrupted proteostasis are likely downstream events in manganese neurotoxicity. Our study shows that the zebrafish slc39a14<sup>U801</sup> loss-of-function mutant is a powerful model to elucidate the mechanistic basis of diseases affected by manganese dyshomeostasis.

**Introduction**

SLC39A14 is a manganese (Mn) uptake transporter essential for the maintenance of Mn homeostasis (Thompson and Wessling-Resnick, 2019). Mutations in SLC39A14 impair cellular Mn uptake and result in systemic Mn overload characterised by significant hypermanganesemia and neurodegeneration (Tuschl et al., 2016; Juneja et al., 2018; Marti-Sanchez et al., 2018; Rodan et al., 2018; Zeglam et al., 2018). In patients, subsequent accumulation of Mn in the globus pallidus, a component of the basal ganglia involved in motor control, leads to rapidly progressive dystonia-parkinsonism with onset in early childhood, a condition known as Hypermanganesemia with Dystonia 2 (HMNDYT2, OMIM # 617013). In a small number of patients, treatment has been attempted with Mn chelation using intravenous disodium calcium edetate (Na₂CaEDTA) similar to a protocol established for HMNDYT1 (OMIM # 613280) caused by mutations in SLC30A10, a Mn exporter required for biliary excretion of Mn (Tuschl et al., 1993; Tuschl et al., 2012). MRI brain imaging of patients with either disorder are indistinguishable; hyperintensity of the basal ganglia, particularly the globus pallidus, and the white matter on T1-weighted imaging is a hallmark of both disorders (Tuschl et al., 2012; Tuschl et al., 2016). While patients with HMNDYT1 show significant improvement of neurological symptoms upon treatment initiation with stabilisation of the disease over many years (Tuschl et al., 2008; Tuschl et al., 2012), individuals with HMNDYT2 have variable treatment response, some even with worsening of their movement disorder (Tuschl et al., 2016; Marti-Sanchez et al., 2018). Consequently, the reasons for the difference in treatment response are poorly understood.

Although an essential trace metal, excess Mn has long been known to act as a neurotoxicant. Environmental Mn overexposure leads to preferential Mn accumulation in the globus pallidus similar to that observed in inherited Mn transporter defects, and causes manganese, a Parkinsonian movement disorder characterised by bradykinesia, akinetic rigidity, and dystonia, accompanied by psychiatric disturbances (Blanc, 2018; Chen et al., 2018). Despite its recognised role in neurodegenerative disease processes, we lack a deeper understanding of the mechanisms of Mn related neurotoxicity. The clinical similarities between manganese and Parkinson’s disease (PD) suggest that dopaminergic signalling is impaired upon Mn toxicity. However, in manganese, dopaminergic neurons within the substantia nigra are intact.
and response to L-DOPA is poor (Koller et al., 2004). Glutamatergic excitotoxicity as well as altered gamma-aminobutyric acid (GABA) signalling have also been proposed to underlie Mn associated neurodegeneration (Caito and Aschner, 2015). Indeed, Mn toxicity is likely mediated by a number of processes including oxidative stress, impaired mitochondrial function, protein misfolding and aggregation, and neuroinflammation (Martinez-Finley et al., 2013; Tjalkens et al., 2017).

We have recently established and characterised a zebrafish loss-of-function mutant slc39a14U801−/− that closely resembles the human phenotype with systemic accumulation of Mn, particularly in the brain (Tuschl et al., 2016). Homozygous mutants develop increased susceptibility to Mn toxicity and impaired locomotor behaviour upon Mn exposure. Mn levels can be lowered through chelation with Na₂CaEDTA similar to what is observed in human patients (Troche et al., 2016).

In this study, we used RNA sequencing on individual larvae from an in-cross of heterozygous slc39a14U801 zebrafish to identify novel potential targets of Mn toxicity. Furthermore, we determined the transcriptional signature elicited in response to MnCl₂ treatment in mutant and sibling fish. Our results provide evidence that, in addition to Mn neurotoxicity, partial Mn deficiency that corrects upon Mn treatment is a prominent feature of slc39a14 loss-of-function. We also determined that Ca²⁺ dyshomeostasis is a likely key event in both Mn deficiency and overload. Mn neurotoxicity appears to be further associated with activation of the unfolded protein response (UPR), oxidative stress, mitochondrial dysfunction, apoptosis and autophagy, and disruption of lysosomes and proteostasis.

Materials and Methods

Zebrafish husbandry

Zebrafish were reared on a 14/10 h light/dark cycle at 28.5°C. Embryos were obtained by natural spawning and staging was performed according to standard criteria (Kimmel et al., 1995). Previously generated slc39a14U801 loss-of-function zebrafish and their siblings were used for all experiments (Tuschl et al., 2016). Ethical approval for zebrafish experiments was obtained from the Home Office UK under the Animal Scientific Procedures Act 1986.

Preparation of larvae for RNA and DNA extraction

The progeny of a single in-cross of slc39a14U801+/− fish were raised under standard conditions. At 2 dpf, the larvae were split into two groups and one group was exposed to MnCl₂ added to the fishwater at a concentration of 50 µM. After 72 hours of exposure (at 5 dpf) single larvae were collected in the wells of a 96 well plate, immediately frozen on dry ice and stored at -80°C. For sequencing, frozen embryos were lysed in 100 µl RLT buffer (Qiagen) containing 1 µl of 14.3M beta mercaptoethanol (Sigma). The lysate was allowed to bind to 1.8 volumes of Agencourt RNAClean XP (Beckman Coulter) beads for 10 mins. The plate was then applied to a plate magnet (Invitrogen) until the solution cleared and the supernatant was removed without disturbing the beads. While still on the magnet the beads were washed three times with 70% ethanol and total nucleic acid was eluted from the beads as per the manufacturer’s instructions. Nucleic acid samples were used for genotyping of individual larvae by KASP assay (LGC Genomics) according to the manufacturer’s instructions and the following primers: wild-type allele 5’ GGCACATAATAATCCTCCATGGG 3’, mutant allele 5’ GGGACATAATACCTCCTGATGG 3’ and common primer 5’ CCCTGTATGTAGGCCTTCGGGT 3’. After DNase treatment, RNA was quantified using either Qubit RNA HS assay or Quant-iT RNA assay (Invitrogen).
Transcript counting

DeTCT libraries were generated as described previously (Collins et al., 2015). Briefly, 300 ng of RNA from each genotyped sample was fragmented and bound to streptavidin beads. The 3’ ends of the fragmented RNA were pulled down using a biotinylated polyT primer. An RNA oligo containing the partial Illumina adapter 2 was ligated to the 5’ end of the bound fragment. The RNA fragment was eluted and reverse transcribed using an anchored oligo dT reverse transcriptase primer containing one of the 96 unique index sequences and part of the Illumina adapter 1. The Illumina adapters were completed during a library amplification step and the libraries were quantified using either the BioPhotometer (Eppendorf) or PheraStar (BMG Labtech). This was followed by size selection for an insert size of 70–270 bases. Equal quantities of libraries for each experiment were pooled, quantified by qPCR and sequenced on either HiSeq 2000 or HiSeq 2500.

Sequencing data were analysed as described previously (Collins et al., 2015). Briefly, sequencing reads were processed with the DeTCT detag_fastq.pl script and aligned to the GRCz11 reference genome with BWA 0.5.10 (Li and Durbin, 2009). The resulting BAM files were processed using the DeTCT pipeline, which results in a list of regions (for simplicity referred to as genes in the Results) representing 3’ ends, together with a count for each sample. These counts were used for differential expression analysis with DESeq2 (Love et al., 2014). Each region was associated with Ensembl 95 (Yates et al., 2020) gene annotation based on the nearest transcript in the appropriate orientation. False positive 3’ ends, representing, for example, polyA-rich regions of the genome, were filtered using the DeTCT filter_output.pl script with the—strict option.

Gene sets were analysed using the Cytoscape plugin ClueGO (Bindea et al., 2009) for gene ontology (GO) enrichment and Ontologizer (Bauer et al., 2008) for Zebrafish Anatomy Ontology (ZFA) enrichment.

Quantitative real time PCR (qRT-PCR)

RNA extraction from 30 zebrafish larvae from the same genotype (homozygous mutant or wild-type) was performed using the TRIzol reagent (Invitrogen) according to the recommended protocol. DNA extraction was performed using the HotSHOT method (Truett et al., 2000). qRT-PCR was performed using GoTaq qPCR Master Mix (Promega) according to the recommended protocol. All samples were run in triplicates. qRT-PCR was carried out on a CFX96 Touch Real-Time PCR Detection System (BioRad). Only primer pairs with R2 values >0.99 and amplification efficiencies between 95% and 105% were used. Relative quantification of gene expression was determined using the 2^ΔΔCt method, with elongation factor 1α (ef1α) as a reference gene (Livak and Schmittgen, 2001). The following primer sequences were used: ef1α forward 5’GACTTTCAGGCTGACTTG3’, reverse 5’ACGATACGTTCCTCACCTCC3’; bdnf forward 5’AGATCGGCTGGCGCTTAA3’, reverse 5’CATTGTGTACACTATCTGCC3’; gnat2 forward 5’GCTGGCAGACGTCATCAAAA3’, reverse 5’CTCCTTGCAGAGGTAGTACG3’; hspa5 forward 5’GCTGGGCTGAATGTCATGAG3’, reverse 5’CAGCAGAGACACGTCAAAGG3’; pde6h forward 5’GCTGTCATTTCGTCTCCTC3’, reverse 5’GACCATCGTGTTACTTCTCC3’; prph2b forward 5’GCCCTGTGTCTCTACTATGG3’, reverse 5’CTCCTGGGATTCTGCTGGT3’.

Optokinetic response (OKR)

The OKR was examined using a custom-built rig to track horizontal eye movements in response to whole-field motion stimuli. Larvae at 4 dpf were immobilised in 1.5% agarose in a 35 mm petri dish and analysed at 5 dpf. The agarose surrounding the eyes was removed to allow normal eye movements. Sinusoidal gratings with spatial frequencies of 0.05, 0.1, 0.13 and 0.16 cycles/degree were presented on a cylindrical diffusive screen 25 mm from the centre of the fish’s head. Gratings had a constant velocity of 10 degrees/second and changed direction and/or spatial frequency every 20 seconds. Eye movements were tracked under
infrared illumination (720 nm) at 60 Hz using a Flea3 USB machine vision camera and custom-written software. A custom-designed Matlab code was used to determine the eye velocity (degrees per second).

**Retinal histology**

5dpf larvae were fixed in 4% PFA overnight at 4°C. Dehydration was achieved by a series of increasing ethanol concentrations in PBS (50%, 70%, 80%, 90%, 95% and 100% ethanol). After dehydration larvae were incubated in a 1:1 ethanol Technovit 7100 solution (1% Hardener 1 in Technovit 7100 basic solution) for 1 h followed by incubation in 100% Technovit solution overnight at room temperature (Heraeus Kulzer, Germany). Larvae were then embedded in plastic moulds in Technovit 7100 polymerization medium and dried at 37°C for 1 h. Sections of 3 µm thickness were prepared with a microtome, mounted onto glass slides, and dried at 60°C. Sections were stained with Richardson (Romeis) solution (0.5% Borax, 0.5% Azur II, 0.5% Methylene Blue) and slides were mounted with Entellan (Merck, Darmstadt, Germany). Images were taken in the brightfield mode of a BX61 microscope (Olympus).

**Experimental design and statistical analyses**

Animals were divided into four experimental groups: unexposed homozygous slc39a14<sup>U801−/−</sup> mutants and their siblings (wild-type and heterozygous genotypes), and MnCl<sub>2</sub> exposed homozygous slc39a14<sup>U801−/−</sup> mutants and their siblings (wild-type and heterozygous genotypes). For the DeTCT data, an equal number of wild-type and heterozygous embryos were selected (see Fig. 1 for numbers of embryos for each experimental group). Embryos were all derived from a single cross to minimise the amount of biological variance not caused by the experimental conditions (i.e. genotype and Mn exposure). One wild-type Mn-exposed embryo was excluded from the data after visual inspection of the Principal Component Analysis as it did not group with any of the other samples. DESeq2 was used for differential expression analysis with the following model: ~ genotype + treatment + genotype:treatment. This models the observed counts as a function of the genotype (homozygous vs siblings) and the treatment (Mn exposed vs unexposed) and an interaction between the two and tests for significant parameters using the Wald test with a p value threshold of 0.05. For qRT-PCR and OKR analysis ANOVA with Tukey post-hoc testing was used to determine statistical significance, using the GraphPad Prism software (version 5). For GO term analysis, the settings for ClueGO were as follows: a right-sided hypergeometric test (enrichment only) was used with the Bonferroni step-down (Holm-Bonferroni) correction for multiple testing and terms with corrected p values >0.05 were discarded. For ZFA enrichment analysis, the Ontologizer Parent-Child-Union calculation method was used with Bonferroni correction.

**Transcription factor motif analysis**

Transcription factor motif enrichment was performed using HOMER's findMotifs.pl tool (v4.10.3) with default settings (Heinz et al., 2010). The GRCz11 promoter set used was created with HOMER's updatePromoters.pl tool based on RefSeq genes from -2000 bp to 2000 bp relative to the TSS.

**Results**

Transcriptome analysis of slc39a14<sup>U801−/−</sup> mutants identifies increased sensitivity to Mn toxicity and highlights additional Mn deficiency effects in homozygous mutants

To investigate the transcriptional profiles of slc39a14<sup>U801−/−</sup> mutants in the absence and presence of Mn treatment, embryos from a heterozygous in-cross were split into two groups and either raised under standard conditions (later referred to as unexposed), or treated with 50 µM MnCl<sub>2</sub> from 2 until 5 days post fertilisation (dpf) (Fig. 1A). We have previously shown that this concentration elicits a locomotor phenotype in homozygous mutant larvae that is absent in siblings (Tuschl et al., 2016). We then carried out transcriptional profiling of individual 5 dpf larvae using 3' tag sequencing (Collins et al., 2015). Principal Component Analysis (PCA) shows that the samples cluster according to genotype and treatment status (Fig. 1B).
Analysis of differentially expressed genes between the four conditions produced three large sets of genes where each set had a characteristic expression profile. The first set are genes that are differentially expressed in MnCl₂ exposed siblings compared with unexposed siblings (Fig. 1C, Mn toxicity) and represent a response to an increased concentration of Mn in the embryos. The second set contains genes that show increased sensitivity to Mn in slc39a14U801⁻/⁻ mutants. These are defined as genes that are differentially expressed in MnCl₂ exposed mutants compared with unexposed siblings, but not differentially expressed in unexposed mutants compared to unexposed siblings or exposed siblings with unexposed siblings (Fig. 1D, Increased sensitivity). The third set is composed of genes that are differentially expressed in unexposed mutants compared with unexposed siblings (Fig. 1E, Mutant effect). We will now consider these three groups of genes in turn (Table 1).
Fig. 1. DeTCT analysis identifies three groups of differentially expressed genes.

(A) Diagram of the experiment. Embryos from a slc39a14<sup>U807</sup> heterozygous in-cross were either exposed to 50 µM MnCl<sub>2</sub> or left unexposed from 2 to 5 dpf.

(B) Principal Component Analysis of the samples. Principal component (PC) 1 is plotted on the x-axis and PC2 on the y-axis. Samples belonging to the same condition group together. Unexposed sibling embryos are light blue and MnCl<sub>2</sub> exposed ones are dark blue. Unexposed mutants are coloured light red and exposed mutants are dark red.

(C) Group 1 (Mn toxicity) genes are defined as those with a significant difference between exposed and unexposed siblings (red bar with asterisk). Example plot of normalised counts for the soul5 gene. The colour scheme for C–E is the same as in (B).

(D) Group 2 (Increased sensitivity) genes are defined as those with a significant difference between exposed mutants and unexposed siblings (red bar with asterisk) without significant differences in either unexposed mutants or exposed siblings when compared to unexposed siblings (black bars labelled NS).

(E) Group 3 (Mutant effect) is defined as genes with a significant difference between unexposed mutants and unexposed siblings (red bar with asterisk).
<table>
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<tr>
<th>Mn toxicity</th>
<th>Increased sensitivity of slc39a14&lt;sup&gt;Ubo1&lt;/sup&gt;/&lt;sup&gt;-&lt;/sup&gt; mutants to MnCl&lt;sub&gt;2&lt;/sub&gt; treatment</th>
<th>Mutant effect changes in slc39a14&lt;sup&gt;Ubo1&lt;/sup&gt;/&lt;sup&gt;-&lt;/sup&gt; mutants</th>
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<td>Neuronal differentiation/growth</td>
<td>Glutamate neurotransmission slc1a2a, slc1a2b, slc1a8a, nsg2, prrt1</td>
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<td>GABA neurotransmission</td>
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<td>Cell-cell adhesion - Ca&lt;sup&gt;2+&lt;/sup&gt; death 24b, ctnn1b, pcdh1a, pcdh2g17, pcdh7b, pcdh9, pcdh10a, pcdh17, pcdh19</td>
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<td>Glutamate neurotransmission</td>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt; homeostasis</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; homeostasis atp2a2b, atp2b1b, calr3, canx, camk1ga, camk2g1, camkva, capn7, dct, icn, ncaldb, pcdh7b, ppp3r1a, rgn, s100b, scpp1, tnm2a4</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt; homeostasis atp2a1, atp2b3b, cacinb4b, kcnma1a, kcnma1b, kcalmb, kcalmb3, calm3a, calm3b, strm4</td>
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<td>Inhibition of α-synuclein aggregation</td>
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<td>Unfolded protein response atf3, atf4b, atf6, derl1, dnaq11, herpud1, hisp5, hsdp1, hspe1, sylvn1, xbp1</td>
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<td>Connective tissue</td>
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<td>Lipid metabolism</td>
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<td>Autophagy glikp2l, hmgln2, rubcn</td>
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<td>Thyroid metabolism</td>
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<td>Ribosomal function</td>
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<td>Ubiquitination / proteostasis outd5a, rer1, ube23b, ubqln4, ubtd1a, usp9, usp10, usp21</td>
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<td>Ribosomal function &amp; translation rrp8, rrp12, rlp2, rps7, rps20, mrrps30, eif1axb, eif4a1a, eif4bb, eif4c1, eif4g1a, eif4h, eif5b, aars, cars, farsa, fars, kars, larsb, mars, nars, yars</td>
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<td>Lysosomal function</td>
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<td>Lysosomal function cisl, ctek, ctsla, ctsli, lgmn</td>
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<td>Akt/PI3K/mTOR signalling</td>
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<td>Ca&lt;sup&gt;2+&lt;/sup&gt; homeostasis atp2a1</td>
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<td>Glucconeogenesis</td>
<td>gapdh, gapdhs, pkfb3, pkma</td>
<td>Glycosylation alg2, dpmp1, gpaa1, nus1, gpagp2</td>
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<td>atp5l, ckmt2a, mrrps30, nfu1, suclg1, tromn6</td>
<td>Extracellular matrix fn1b, lamb1b, vmb</td>
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Table 1. Differentially expressed genes grouped by function. Full lists in Supplementary Table 1.

Red, increased gene expression. Blue, reduced gene expression.
Mn toxicity causes differential gene expression independent from the genotype

MnCl₂ treatment caused differential expression of 328 genes independent of the genotype (comparing MnCl₂ exposed siblings and unexposed siblings) (Fig. 2A, Table 1 and Supplementary Table 1). Among them is brain-derived neurotrophic factor (bdnf) encoding a protein that is known to be altered upon Mn exposure (Zou et al., 2014). In addition, bdnf expression is also diminished in untreated mutants compared to siblings (Fig. 2B). Given that slc39a14Δ/Δ mutants show evidence of Mn toxicity already at 5 dpf (increased total Mn and reduced locomotor activity), this suggests that bdnf expression is a sensitive read-out for Mn toxicity. Mn associated suppression of BDNF signalling has been linked to diminished numbers of parvalbumin positive cells, mainly GABAergic interneurons (Fairless et al., 2019). Indeed, we find Parvalbumin encoding genes differentially expressed upon Mn exposure in mutants as well as siblings (pvalb2, pvalb8) and in treated mutants only (pvalb1). However, parvalbumin mRNA expression is upregulated in response to Mn in mutants and siblings, which is unexpected given the previously reported link to reduced numbers of Parvalbumin positive cells.

Among other brain-expressed genes affected by MnCl₂ exposure in siblings are some involved in synaptic vesicle function (rims2b, stxbp1a, sv2a, sybp, syt9a), and genes encoding the metabotropic glutamate receptor (grm8a), β-synuclein (sncb) and ephrin-B membrane proteins (efnb1, efnb2a). Reduced ephrin-B levels have been linked to the pathophysiology of Alzheimer’s disease (AD) (Mroczko et al., 2018).

Mn is important for connective tissue integrity and bone mineralisation as a constituent of metalloenzymes and an enzyme co-factor (Sirri et al., 2016; Zofkova et al., 2017). Accordingly, our transcriptome analysis confirms that Mn exposure in zebrafish leads to reduced expression of multiple connective tissue related genes (col2a1b, col4a5, col9a1a, col9a2, col11a2, dcn, fbn2b, matn1).

Analysis of annotations to Gene Ontology (GO) terms shows enrichments of terms related to lipid metabolism (apoa4b.2, apoa4a, apoea), blood cell development (alas1, fech, soul5; Fig. 1C) and translation (35 ribosomal protein encoding genes) (Fig. 2C; Supplementary Table 2).

Mn has previously been shown to interfere with heme-enzyme biogenesis and protein synthesis (Kaur et al., 2017; Chino et al., 2018; Hernandez et al., 2019).
Fig. 2. Manganese overexposure causes neurotoxicity and metabolic defects in wild-type zebrafish.

(A) Heatmap of the expression of all 328 genes with a significant difference between exposed and unexposed siblings (Group 1 - Mn toxicity, Supplementary Table 1). Each row represents a different gene and each column is a sample. Mutant embryos are displayed for completeness although the group of genes is defined by the response in siblings only. The normalised counts for each gene have been mean centred and scaled by dividing by the standard deviation.

(B) Plot of the normalised counts for each sample of a gene (bdnf) in Group 1. Unexposed sibling embryos are light blue and MnCl$_2$ exposed ones are dark blue. Unexposed mutants are coloured light red and exposed mutants are dark red.

(C) Enrichment of Gene Ontology (GO) terms associated with the genes in (A). Diagram produced using the Cytoscape ClueGO App. Nodes represent enriched GO terms and edges connect GO terms that have annotated genes in common. Different components of the network are coloured according to the categories labelled on the diagram.

slc39a14$^{U801-/-}$ mutants show increased sensitivity to MnCl$_2$ treatment compared to siblings.

Our analysis showed that 613 genes are differentially expressed in MnCl$_2$ exposed mutants compared with unexposed siblings, with no significant expression changes in either unexposed mutants or exposed siblings. Therefore, these are genes that show increased sensitivity to MnCl$_2$ exposure in slc39a14$^{U801-/-}$ mutant larvae (Fig. 3A). 15% (95/613) of these genes also have a significant genotype-treatment interaction effect meaning that there is a synergistic effect on expression of treating mutant embryos with MnCl$_2$ – i.e. the combined estimated effects of genotype and MnCl$_2$ treatment alone are significantly less than the estimated log2 fold change for MnCl$_2$ exposed mutants when compared to unexposed siblings (Fig. 3B, Table 1 and Supplementary Table 1). The remaining genes (518/613) show expression changes consistent with additive effects of the sub-significance threshold responses to genotype and MnCl$_2$ exposure alone (Fig. 3C). Results from the transcriptome analysis were validated by qRT-PCR for a subset of six genes (bdnf, gnat2, hspa5, opn1mw2, pde6h, prph2b) using RNA extracted from equivalent embryos in a different experiment (Fig. 3D–E, Supplementary Fig. 1 and Supplementary Table 3). Changes in gene expression observed by qRT-PCR for all six genes were consistent with the results obtained from transcript counting (compare, for instance, Fig. 1D with Fig. 3E and Fig. 3B with Fig. 3D).

Enrichment of zebrafish anatomy (ZFA) terms shows that genes differentially expressed upon MnCl$_2$ exposure in slc39a14$^{U801-/-}$ mutants are disproportionately expressed in the eye and nervous system (Fig. 3F; Supplementary Table 4). This is confirmed by the enrichment of GO terms such as visual perception and phototransduction. Also enriched are terms related to the ribosome, translation and the unfolded protein response (UPR) suggesting effects on protein production and folding (Fig. 3G and Supplementary Table 2).
Fig. 3. Effect of Mn treatment in slc39a14<sup>U801<sup>mutants.</sup></sup>

(A) Heatmap of the expression of all genes (613) with a significant difference between exposed mutant and unexposed sibling embryos without significant treatment or genotype effects. The heatmaps are split into genes that show either synergistic or additive effects of the individual genotype and treatment effects. Each row represents a different gene and each column is a sample. The normalised counts for each gene have been mean centred and scaled by dividing by the standard deviation.

(B) Example of a gene (hspa5) with a synergistic effect of treatment and genotype. The difference between the exposed mutants and unexposed siblings cannot be explained by adding together the separate effects of Mn treatment and the slc39a14<sup>U801</sup> mutation. Unexposed sibling embryos are light blue and MnCl<sub>2</sub> exposed ones are dark blue. Unexposed mutants are coloured light red and exposed mutants are dark red.
(C) Example of a gene (pde6c) that has an additive effect of treatment and genotype. The two sub-threshold effects of treatment and genotype produce the difference between exposed mutants and unexposed siblings when added together. Colour scheme as in (B).

(D–E) qRT-PCR shows comparable gene expression changes as for the single embryo sequencing dataset. The individual samples are displayed as fold change relative to the mean value for unexposed siblings and the mean and 95% confidence intervals for each condition are in orange. (D) hspa5. Compare with (B). (E) pde6ha. Compare with Fig. 1D.

(F) Enrichment Map network of the Zebrafish Anatomy Ontology (ZFA) enrichment results. Each node represents an enriched term and the edges join nodes that have overlapping genes annotated to them. The width of each edge is proportional to amount of overlap, nodes are coloured by -log10(adjusted p value) and the size represents the number of significant genes annotated to the term.

(G) ClueGO network diagram of the enrichment of Gene Ontology (GO) terms. Nodes represent enriched GO terms and edges connect nodes that share annotations to the significant genes. Different components of the network are coloured according to the categories as labelled on the diagram.

**Increased sensitivity of slc39a14<sup>U801-/-</sup> mutants to MnCl<sub>2</sub> treatment leads to Mn neurotoxicity**

Enriched ZFA terms identified in MnCl<sub>2</sub> exposed slc39a14<sup>U801-/-</sup> mutants that are not present in siblings confirm a high number of differentially expressed genes in the nervous system (Supplementary Table 4) consistent with the known role of Mn in neurotoxicity. Differentially expressed genes include several related to glutamatergic, GABAergic and dopaminergic signalling similar to previous studies that demonstrate impaired neurotransmitter signalling as a key event in Mn neurotoxicity (Marreilha Dos Santos et al., 2011). Genes with a link to glutamatergic circuitry include slc1a2a and slc1a2b, encoding the glutamate transporter EAAT2, and slc1a8a, encoding a glutamate transporter present in teleosts only (Gesemann et al., 2010; Karki et al., 2015). Two genes required for the regulation of ionotropic AMPA type glutamate receptors (AMPA) (nsg2, prrt1) show diminished expression in MnCl<sub>2</sub> treated mutants (Chander et al., 2019; Troyano-Rodriguez et al., 2019).

Furthermore, we observe increased expression of slc6a11b, encoding a GABA uptake transporter, as well as the parvalbumin encoding gene (pvalb1) present in GABAergic interneurons. Expression of the GABA-A receptor encoding genes gabra6a and gabrb3, and nptxrb, encoding the neuronal pentraxin receptor expressed in parvalbumin positive interneurons (Kikuchihara et al., 2015), is reduced.

Differentially expressed genes associated with dopaminergic signalling include gnb5b and gpr37l1b that interact with neurotransmission via the D2 receptor, and faim2b for which loss-of-function increases susceptibility to dopaminergic neuron degeneration (Octeau et al., 2014; Komnig et al., 2016; Hertz et al., 2019). Furthermore, genes required for presynaptic neurotransmitter release (rims2a, syngr1a, syt17) show reduced expression. A role for astrocyte mediated Mn neurotoxicity and neuroinflammation is suggested by increased expression of the astrocyte related genes atl5a, atl5b and glap.

**Increased sensitivity of slc39a14<sup>U801-/-</sup> mutants to MnCl<sub>2</sub> treatment is associated with gene expression changes affecting calcium and protein homeostasis, and the unfolded protein response**

Mn toxicity is known to cause protein misfolding and aggregation (Angeli et al., 2014; Harischandra et al., 2019b) and, as previously shown for Mn overexposure in C. elegans (Angeli et al., 2014), multiple genes involved in the UPR have increased expression in slc39a14<sup>U801-/-</sup> mutants upon MnCl<sub>2</sub> treatment while siblings appear unaffected (Table 1 and Supplementary table 1). Ca<sup>2+</sup> homeostasis within the endoplasmic reticulum (ER) plays a major role during the UPR and vice versa. Potentially linked to the UPR, over dozen of Ca<sup>2+</sup> associated/dependent genes are differentially expressed (Table 1). In MnCl<sub>2</sub> treated
slc39a14<sup>U801</sup>−/− mutants we observe differential expression of the Ca<sup>2+</sup> ATPase encoding genes atp2a2b (SERCA2) and atp2b1b (PMCA1) as well as increased expression of genes encoding the Ca<sup>2+</sup> chaperones calreticulin 3 (calr3) and calnexin (canx). Activation of the UPR as well as Ca<sup>2+</sup> dyshomeostasis can promote apoptosis and autophagy. Concordantly, genes involved in autophagy and apoptosis are differentially expressed (Table 1). Degradation of misfolded and aggregated proteins occurs via the ubiquitin-proteasome system within the cytosol (Tamas et al., 2014) and MnCl<sub>2</sub> exposed slc39a14<sup>U801</sup>−/− mutants show gene expression changes linked to ubiquitination (Table 1).

Oxidative stress and mitochondrial dysfunction are prominent features of Mn toxicity (Smith et al., 2017; Harischandra et al., 2019a). Consistent with this observation, essential genes of the thioredoxin/peroxiredoxin system (prdx1, txn, txnrd3) are activated in MnCl<sub>2</sub> exposed slc39a14<sup>U801</sup>−/− mutants. Likewise, genes related to mitochondrial function show differential expression in MnCl<sub>2</sub> treated mutants (Table 1).

As suggested by GO analysis, we observed pronounced expression changes of genes associated with ribosomal function and translation. MnCl<sub>2</sub> treatment of slc39a14<sup>U801</sup>−/− mutants led to differential expression of eleven genes encoding tRNA synthetases, seven genes encoding translation initiation factors and six genes encoding ribosomal proteins. As mentioned above, Mn toxicity independent of the genotype led to differential expression of additional 33 ribosomal protein encoding genes suggesting that protein synthesis is a prominent target of Mn toxicity.

**Increased sensitivity of slc39a14<sup>U801</sup>−/− mutants to MnCl<sub>2</sub> treatment manifests as impaired vision**

30 genes involved in phototransduction were differentially expressed in MnCl<sub>2</sub> exposed mutants but not in siblings (Fig. 4A, Supplementary Table 1). Hence, we further examined the vision of slc39a14<sup>U801</sup>−/− mutants. Raising slc39a14<sup>U801</sup>−/− mutant embryos/larvae on a 14 hour light, 10 hour dark cycle revealed absent visual background adaptation upon MnCl<sub>2</sub> exposure while exposed wild-type larvae and unexposed mutants showed normal pigmentation (Fig. 4B). Visual background adaptation requires normal vision and is therefore impaired in blind larvae (Le et al., 2012). To determine whether slc39a14<sup>U801</sup>−/− larvae develop visual impairment, the optokinetic response (OKR) was analysed in homozygous slc39a14<sup>U801</sup>−/− larvae at 5 dpf after MnCl<sub>2</sub> exposure. Exposed mutant larvae demonstrated a significant reduction in slow phase eye velocity at high spatial frequencies (Fig. 4C). Therefore, as predicted from the observed gene expression changes, Mn exposure leads to visual impairment and subsequent diminished visual background adaptation. Retinal histology appeared normal suggesting functional rather than overt structural deficits (Fig. 4D).
**Fig. 4. slc39a14<sup>U801</sup> loss-of-function mutants develop a visual phenotype upon MnCl<sub>2</sub> exposure.**

(A) Schematic showing the process of phototransduction (Kaupp and Seifert, 2002) with differentially expressed genes observed in MnCl<sub>2</sub> exposed slc39a14<sup>U801<sup>−/−</sup> mutants in italics. cGMP, cyclic guanosine monophosphate. CNG, cyclic nucleotide gated non-selective cation channels. GC, guanylyl cyclase. GCAP, guanylate cyclase activating protein. PDE, phosphodiesterase. GRK, G-protein coupled receptor kinase. GAP, GTPase activating protein.
(B) Dorsal views of wild-type siblings (slc39a14\textsuperscript{U801+/+}, on the left) and slc39a14\textsuperscript{U801−/−} larvae (on the right) at 5 dpf unexposed and exposed to 50 \(\mu\)M MnCl\(_2\). * indicates abnormal visual background adaptation. Scale bar 500 \(\mu\)m.

(C) Graph showing the OKR (average of both eyes) of slc39a14\textsuperscript{U801−/−} larvae unexposed (dark green squares) and exposed to 50 \(\mu\)M MnCl\(_2\) (light green circles). Data are presented as mean ± s.e.m. from five independent experiments. (**p<0.01; *** p<0.001).

(D) Histologic analysis of retinal sections stained with Richardson-Lowry reagent (green circles) and exposed to 50 \(\mu\)M MnCl\(_2\) (dark green squares). Data are presented as mean ± s.e.m.

Most genes affected in unexposed slc39a14\textsuperscript{U801−/−} mutants are rescued by Mn treatment suggesting Mn deficiency

When compared to unexposed siblings, 266 genes show significantly different expression due to the U801 mutation alone (unexposed mutants versus unexposed siblings) (Fig. 5A; Supplementary table 1). Expression of 12% of these genes (31/266) is also significantly different between MnCl\(_2\) exposed mutants and unexposed siblings (Fig. 5B). Seven of these genes overlap with those differentially expressed in siblings upon MnCl\(_2\) exposure suggesting that these genes are sensitive targets of Mn toxicity (alas1, atp2a1, bdnf, crim1, dio3b, dip2ca, rims2b). However, the majority (88%, 235/266) of differentially expressed genes in unexposed mutants are not significantly differentially expressed when comparing MnCl\(_2\) exposed mutants and unexposed siblings (Fig. 5C). This suggests that the U801 mutation creates Mn deficiency leading to gene expression changes that are rescued by MnCl\(_2\) treatment towards levels observed in unexposed siblings.

Zebrafish anatomy (ZFA) terms for the nervous system are enriched in this set of genes (Fig. 5D; Supplementary Table 4) and there is an enrichment for the GO terms cell-cell morphology, adhesion and cell-cell interactions (cadm3, cdh24b, cttnb1, fhod3b, fnbp1a, fnbp4, nlg2b, nrcama, nrxn3a, pcdh1a, pcdh2g17, pcdh7b, pcdh9, pcdh10a, pcdh17) (Fig. 5E; Supplementary Table 2). Other brain expressed genes that change upon Mn deficiency include some essential for synaptic function and vesicle formation (snap25a, sv2a, sypb, syt6a, syt9a), neurite and axonal growth (dock3, gas7a, kalma, kalnb, lrrc4c) and potassium channels (kcnc1a, kcnc3a).

In addition, a group of differentially expressed Ca\(^{2+}\) associated genes are rescued by Mn treatment that is different to that observed upon Mn toxicity. These include genes encoding Ca\(^{2+}\) ATPases (atp2a1, atp2b3b), Ca\(^{2+}\) channels (cacnb4b), Ca\(^{2+}\) activated potassium channels (kcnma1a, kcnna1a), calmodulins (calm1b, calm3a) and calmodulin binding proteins (camta1b, strn4). Similarly, expression changes of genes involved in proteostasis and ubiquitination are observed in both Mn deficiency and toxicity, with a distinct affected gene set for each condition (Table 1).
Fig. 5. Exogenous Mn restores normal expression of many genes differentially expressed in unexposed slc39a14<sup>U801^−/−</sup> mutants.

(A) Heatmap of the expression of 266 genes with a significant difference between unexposed mutants and unexposed siblings. Each row represents a different gene and each column is a sample. The normalised counts for each gene have been mean centred and scaled by dividing by the standard deviation.

(B) Plot of normalised counts for the add2 gene. Expression is decreased in both unexposed and MnCl₂ exposed mutant embryos. Unexposed sibling embryos are light blue and Mn-exposed ones are dark blue. Unexposed mutants are coloured light red and exposed mutants are dark red.

(C) Plot of normalised counts for the pcdh7b gene. There are decreased counts in the unexposed mutant embryos that are rescued back to wild-type levels upon 50 µM MnCl₂ treatment. Colour scheme as in (B).

(D) Enrichment Map diagram of the enrichment of Zebrafish Anatomy Ontology (ZFA) terms for the genes differentially expressed in unexposed mutants that are rescued by Mn treatment. Nodes represent enriched ZFA terms and edges connect nodes that share annotations to the significant genes. The width of each edge is proportional to amount of overlap, nodes are coloured by -log<sub>10</sub>[Adjusted p value] and the size represents the number of significant genes annotated to the term.

(E) ClueGO network diagram of the enrichment of Gene Ontology (GO) terms associated with the genes that are rescued by Mn treatment. Nodes represent enriched GO terms and edges connect nodes that share annotations to the significant genes. Different components of the network are coloured according to the categories as labelled on the diagram.
Both Mn toxicity and deficiency in \textit{slc29a14}\textsuperscript{U801/-} mutants target the central nervous system

We analysed the three different gene sets for transcription factor motif enrichment using HOMER (Fig. 6A). The only enriched motifs we could identify were from the largest gene set identified in \textit{slc29a14}\textsuperscript{U801/-} mutants upon MnCl\textsubscript{2} treatment that were unchanged in treated siblings. The motifs included Chop/Atf4 which are part of the unfolded protein response (UPR), as well as HLF, NFIL3 and CEBP:AP1 (Supplementary Table 5). We next examined the enriched Zebrafish Anatomy Ontology (ZFA) terms for each gene set to identify tissue specificity of the observed gene expression changes (Fig. 6B). Whereas differentially expressed genes due to Mn toxicity effects independent of the genotype showed enrichment of ZFA terms primarily associated with liver and gut, the genes with differential expression due to Mn deficiency and increased sensitivity to Mn in \textit{slc29a14}\textsuperscript{U801/-} mutants showed enrichment for the central nervous system (Supplementary Table 4).

\textbf{Fig. 6. Comparative analysis of gene sets.}

(A) Example consensus binding motifs found to be enriched in the promoters of genes that show increased sensitivity to Mn treatment in \textit{slc29a14}\textsuperscript{U801/-} mutants (Group 1). The height of each base represents its frequency at that position in the consensus motif.

(B) Bubble plot of the ZFA enrichment results across the three categories of response. Individual enriched ZFA terms were aggregated to the tissue/organ level. For example, the terms optic cup, retina and photoreceptor cell are all aggregated to the parent term eye. The size of each circle represents the number of individual terms enriched for the particular organ or tissue and they are coloured by the smallest of the p values (-log\textsubscript{10} scaled).

\textbf{Discussion}

Transcriptional profiling of \textit{slc29a14}\textsuperscript{U801} mutant zebrafish has identified distinct gene groups that are differentially expressed in normal physiological conditions and upon MnCl\textsubscript{2} exposure. Consistent with the neurodegenerative phenotype observed in HMNDYT2 patients and the previously described accumulation of Mn in the brain of \textit{slc29a14}\textsuperscript{U801/-} mutants (Tuschl et al., 2016), the majority of differentially expressed genes map to the CNS and the eye. Transcriptome analysis showed that Mn treatment leads to gene expression changes in both \textit{slc29a14}\textsuperscript{U801/-} mutant and sibling zebrafish. Mutant larvae show differential expression of a much greater number of genes upon MnCl\textsubscript{2} treatment that is not observed in treated siblings confirming an increased sensitivity to Mn toxicity. In addition, numerous differentially expressed genes in unexposed \textit{slc29a14}\textsuperscript{U801/-} mutants normalised upon MnCl\textsubscript{2} treatment. This suggests that Mn treatment in \textit{slc29a14}\textsuperscript{U801/-} mutants rescues some of the transcriptomic
changes observed in unexposed mutants. This implies that SLC39A14 loss leads to Mn deficiency in parallel to the observed Mn accumulation.

**Loss of slc39a14 function in zebrafish causes Mn deficiency**

Perhaps the most intriguing observation from the transcriptional profiling was that most differentially expressed genes in unexposed slc39a14<sup>U801-/-</sup> mutants normalised upon MnCl<sub>2</sub> treatment. This indicates that whilst SLC39A14 deficiency leads to systemic Mn accumulation in some locations it also causes deficiency of Mn in some parts of the cell or specific types of cells due to its role as a Mn uptake transporter. One implication from this conclusion is that in patients, Mn chelation treatment would require careful monitoring in order to prevent over-chelation. Reducing Mn availability in parts of the cell may aggravate the neurological disease and lead to further decline. This partial Mn deficiency may explain why chelation therapy in patients with HMNDYT2 is less effective compared to those with HMNDYT1. There are only two individuals out of a dozen patients with HMNDYT2 reported in the literature who had a marked improvement upon Mn chelation (Tuschl et al., 2016; Rodan et al., 2018). Other treatment attempts have been less successful with some patients deteriorating upon Mn chelation (Tuschl et al., 2016; Marti-Sanchez et al., 2018).

The presence of Mn deficiency in slc39a14<sup>U801-/-</sup> mutants suggests that some features of HMNDYT2 may overlap with those observed in SLC39A8 deficiency, an inherited Mn transporter defect leading to systemic Mn deficiency (OMIM #616721). Affected individuals present with intellectual disability, developmental delay, hypotonia, epilepsy, strabismus, cerebellar atrophy and short stature (Boycott et al., 2015; Park et al., 2015). However, HMNDYT2 does not lead to any of these features aside from cerebellar atrophy described in some patients. SLC39A8 deficiency is also associated with dysglycosylation as Mn acts as a cofactor for the β-1,4-galactosyltransferase in the Golgi. However, transferrin glycosylation in HMNDYT2 is normal suggesting that Mn levels within the Golgi are not reduced (Tuschl et al., 2016).

The majority of differentially expressed genes in unexposed slc39a14<sup>U801-/-</sup> mutants that correct upon Mn treatment map to the CNS. As for Mn toxicity, several differentially expressed genes link to Ca<sup>2+</sup> homeostasis and binding, however, these are different to those identified upon Mn overload. It is plausible that altered Mn levels, in both Mn deficiency and overload, result in Ca<sup>2+</sup> dyshomeostasis. Expression of multiple genes encoding Ca<sup>2+</sup> dependent cell-cell adhesion and interaction proteins, particularly protocadherins and formin related genes, is reduced in unexposed slc39a14<sup>U801-/-</sup> mutants. Protocadherins are mainly expressed in the CNS where they are required for normal neural circuitry activity and regulate synaptic function (Kim et al., 2011). Loss of protocadherin function in mice has been previously associated with neurodegeneration (Hasegawa et al., 2016). Formins are required for stabilisation of E-cadherins (Rao and Zaidel-Bar, 2016) which may link the changes observed in (proto)cadherin expression with that of formin-associated genes. In addition, a number of genes required for Ca<sup>2+</sup> triggered synaptic vesicle exocytosis was differentially expressed. Interestingly, synaptotagmin 1 can bind Ca<sup>2+</sup> and Mn<sup>2+</sup> in the same manner (Ubach et al., 1998). Mn dyshomeostasis may therefore directly affect neurotransmitter release.

**Unexposed slc39a14<sup>U801-/-</sup> mutants as well as MnCl<sub>2</sub> treated mutants and siblings show evidence of Mn neurotoxicity**

The mechanisms underlying Mn neurotoxicity are heterogeneous suggesting an extensive role for Mn in brain pathobiology. Occupational manganism is associated with lower plasma BDNF levels (Zou et al., 2014), and Mn treatment in mice and rats reduces BDNF levels (Stansfield et al., 2014; Zhu et al., 2019). Indeed, bdnf expression is reduced in untreated slc39a14<sup>U801-/-</sup> mutants as well as MnCl<sub>2</sub> exposed siblings confirming that bdnf expression is a sensitive readout of Mn neurotoxicity. BDNF promotes neuronal cell survival, neurite growth and cell migration, and as such is required for the postnatal growth of the striatum (Rauskolb et al., 2010).
In addition, Mn overexposure has previously been shown to disrupt neurotransmitter release via interaction with the SNARE complex which is mediated by increased intracellular Ca\(^{2+}\) levels and subsequent activation of calpain, a Ca\(^{2+}\)/Mn\(^{2+}\)-activated neutral protease (Wang et al., 2018). Our results provide evidence that Mn neurotoxicity in slc39a14\(^{U801}\)-/ mutants affects expression of genes encoding parts of the presynaptic neurotransmitter release machinery such as rims2a, rims2b, syngr1a and syt17 as well as calpain (capn7).

The neuronal subtypes affected by Mn neurotoxicity remain subject of debate. Consistent with previous reports we observe altered expression of genes involved in glutamatergic, GABAergic and dopaminergic neurotransmission in MnCl\(_2\) treated slc39a14\(^{U801}\)-/ mutants (Marreilha Dos Santos et al., 2017). Mn overexposure has been linked to impaired uptake of glutamate from the synaptic cleft resulting in glutamatergic excitotoxicity (Erikson et al., 2008; Avila et al., 2010). In keeping with this finding, genes encoding glutamate uptake transporters (slc1a2a, slc1a2b, slc1a8a) as well as some required for the regulation of AMPA-type glutamate receptors (nsg2, prrt1) (Chander et al., 2019; Troyano-Rodriguez et al., 2019) are differentially expressed in MnCl\(_2\) exposed slc39a14\(^{U801}\)-/ mutants. SLC1A2 encodes the glutamate uptake transporter EAAT2 that is expressed on astrocytes and known to be downregulated upon MnCl\(_2\) exposure, subsequently leading to glutamate excitotoxicity (Karki et al., 2015).

In HMNDYT2 patients, Mn preferentially accumulates in the globus pallidus, a region that is particularly rich in GABAergic projections (Sidoryk-Wegrzynowicz and Aschner, 2013; Tuschl et al., 2016). In MnCl\(_2\) treated slc39a14\(^{U801}\)-/ mutants expression of genes encoding the GABA-A receptor (gabra6a, gabrb3) and the GABA reuptake transporter (slc6a11b) is reduced. This is consistent with studies in rats where Mn exposure leads to diminished GABA-A receptor mRNA expression and interferes with GABA uptake in astrocytes (Fordahl and Erikson, 2014; Ou et al., 2017). Increased expression of genes encoding parvalbumin (pvalb1, pvalb2 and pvalb8) in slc39a14\(^{U801}\)-/ mutants and siblings upon MnCl\(_2\) treatment may be consistent with previous findings suggesting that GABAergic interneurons are a target of Mn neurotoxicity (Kikuchihara et al., 2015). Parvalbumin, a Ca\(^{2+}\) binding protein, can also bind Mn\(^{2+}\) with high affinity (Nara et al., 1994). Mn may therefore interact with parvalbumin directly or via changes in Ca\(^{2+}\) homeostasis. Mn exposure in mice leads to a reduction of parvalbumin positive cells likely due to suppression of BDNF signalling (Kikuchihara et al., 2015). Parvalbumin positive interneurons also express neuronal pentraxins and the neuronal pentraxin receptor. Pentraxins have previously been shown to play a role in neuroinflammation in PD and AD (Yin et al., 2009). Indeed, expression of nptxrb encoding the neuronal pentraxin receptor is reduced in slc39a14\(^{U801}\)-/ mutants upon MnCl\(_2\) treatment.

Because manganism resembles Parkinson’s disease to some extent (both cause an akinetic movement disorder, albeit, with distinct clinical features) it seemed plausible that dopaminergic neurons are affected by Mn neurotoxicity. Indeed, several studies have shown dopaminergic neurodegeneration upon Mn exposure (Ijomone et al., 2016). However, transcriptome analysis of slc39a14\(^{U801}\)-/ mutants identified changes in only three genes linked to dopaminergic signalling. gnb5b and gpr37l1b interact with neurotransmission via the D2 receptor, and loss-of-function of faim2b leads to increased susceptibility to dopaminergic neuron degeneration (Octeau et al., 2014; Konnig et al., 2016; Hertz et al., 2019). Therefore, it appears likely that interference with genes encoding proteins involved in dopaminergic circuitries is not the primary pathogenesis in slc39a14\(^{U801}\)-/ mutants.

Neuroinflammation has been linked to Mn neurotoxicity supported by the observation that Mn predominantly accumulates in astrocytes rather than neurons (Tjalkens et al., 2017; Gorojod et al., 2018; Popichak et al., 2018). Indeed, Mn exposure in slc39a14\(^{U801}\) loss-of-function mutants leads to differential expression of the astrocyte related genes atf5a, atf5b and gfap.
Mn toxicity in slc39a14<sup>U801</sup>-/- mutants is associated with calcium dyshomeostasis, activation of the unfolded protein response and oxidative stress

Mn can replace Ca<sup>2+</sup> in its biologically active sites and thereby affect Ca<sup>2+</sup> homeostasis (Kalbitzer et al., 1978; Song et al., 2017). Mn overexposure increases intracellular Ca<sup>2+</sup> concentrations due to disruption of Ca<sup>2+</sup> homeostasis at the mitochondria and the ER (Quintanar et al., 2012) and has previously been linked to neuronal loss and neurodegeneration (Choudhary et al., 2018; Ijomone et al., 2019). Chronically elevated Ca<sup>2+</sup> levels leading to altered cellular signalling and mitochondrial damage is also a hallmark of neurodegeneration in PD (Ludtmann and Abramov, 2018). Indeed, Mn overload in slc39a14<sup>U801</sup>-/- mutants causes significant expression changes of Ca<sup>2+</sup> associated genes. Impaired Ca<sup>2+</sup> homeostasis may directly affect bdnf expression that is modulated by Ca<sup>2+</sup>/CaMK signalling (Liu et al., 2017). Ca<sup>2+</sup> homeostasis is maintained by the ER, the key organelle in regulating proteostasis (Wang et al., 2012). ER stress is clearly evident in MnCl2 exposed slc39a14<sup>U801</sup>-/- mutants as multiple UPR associated genes are upregulated. HOMER analysis also confirms enrichment of the Chop/Atf4 motif in MnCl2 treated mutants. This is consistent with previous studies that show increased expression of ATF6 and HSPA5 as well as increased Xbp1 mRNA splicing in Mn exposed brain slices (Xu et al., 2013). ER stress increases the expression of calcium pumps and chaperones such as calreticulin which help to alleviate protein misfolding while dysfunctional Ca<sup>2+</sup> chaperones cause activation of the UPR (Carreras-Sureda et al., 2018). Calreticulin and calnexin act together as a quality control system that causes retention of misfolded proteins within the ER (McCaffrey and Braakman, 2016). Expression of both genes is increased in MnCl2 exposed slc39a14<sup>U801</sup>-/- mutants.

Generation of reactive oxygen species (ROS) with subsequent oxidative stress and mitochondrial dysfunction is a hallmark of neurodegenerative disorders as well as metal toxicity and contributes to protein misfolding (Gomez and Germain, 2019; Harischandra et al., 2019a). The thioredoxin/peroxiredoxin system required for the reduction of H<sub>2</sub>O<sub>2</sub> protects cells from oxidative stress (Samet and Wages, 2018). Oxidative stress is highlighted by the upregulation of the thioredoxin/thioredoxin reductase and peroxiredoxin system in MnCl2 exposed slc39a14<sup>U801</sup>-/- loss-of-function mutants, similar to previous reports in rats (Taka et al., 2012). Increased ROS generation itself can cause Ca<sup>2+</sup> dyshomeostasis, lysosomal impairment, abnormal protein folding and mitochondrial dysfunction (Gorlach et al., 2015; Harischandra et al., 2019a). ROS leads to oxidation of the thiol group in cysteines of Ca<sup>2+</sup> channels and pumps thereby affecting intracellular Ca<sup>2+</sup> levels (Zhang et al., 2016). Furthermore, ROS cause apoptosis and autophagy via lysosomal membrane permeabilisation and cathepsin release (Gorojod et al., 2017; Wang et al., 2017; Porte Alcon et al., 2018; Zhi et al., 2019). Consistent with this observation, the key autophagy gene rubcn, encoding a beclin 1 interactor and responsible for autophagy initiation (Liu et al., 2019), is upregulated in slc39a14<sup>U801</sup>-/- mutants due to Mn overload. In addition, cathepsin gene expression is altered in MnCl2 treated slc39a14<sup>U801</sup>-/- mutants linking manganese to dysregulation of lysosomal function and autophagy as previously suggested (Zhang et al., 2019).

Mn toxicity interferes with protein synthesis and metabolism

As suggested by GO term enrichment analysis, MnCl2 exposure led to differential expression of multiple genes encoding ribosomal proteins, tRNA synthetases and translation initiation factors in slc39a14<sup>U801</sup>-/- mutants. Interference of Mn with protein synthesis has been identified in yeast where Mn overexposure leads to reduced total tRNA levels and diminished ribosome formation (Hernandez et al., 2019). In addition, MnCl2 exposure in slc39a14<sup>U801</sup>-/- mutants is associated with gene expression changes linked to the Ubiquitination/Proteasome System (UPS). The UPS, essential for protein quality control, is susceptible to oxidative stress (Li et al., 2011; Zhang et al., 2016). Misregulation of the UPS has causally been linked to neurodegeneration in PD (Walden and Muqit, 2017). Heavy metals impair protein folding and promote protein aggregation suggesting that Mn can equally contribute to protein misfolding (Tamas et al., 2014).
Mn toxicity in in *slc39a14* \(^{U801/−}\) zebrafish causes a visual phenotype

Interestingly, transcriptome analysis revealed an unsuspected Mn toxicity effect in *slc39a14* \(^{U801/−}\) zebrafish, a pronounced visual phenotype characterised by impaired visual background adaptation and impaired OKR upon MnCl\(_2\) exposure. To date, retinal Mn toxicity has not been previously reported in affected patients or animal models. Neither environmental overexposure nor systemic Mn accumulation in HMNDYT1 and HMNDYT2 lead to impaired vision in humans. Inherited Mn transporter defects have only recently been reported and it is possible that visual function becomes affected only in later life. Indeed, both Mn uptake transporters, SLC39A8 and SLC39A14, are highly expressed in the retinal pigment epithelium (RPE) (Leung et al., 2008). It has previously been shown that other heavy metals such as cadmium and lead accumulate in ocular tissues, particularly in the RPE (Erie et al., 2005). Mn plays an essential role in retinal function where it is required for normal ultrastructure of the retina (Gong and Amemiya, 1996). Possible differences between the human and zebrafish phenotype may simply be caused by the direct contact of the zebrafish eye with Mn in the freshwater contributing to enhanced ocular Mn uptake and toxicity.

In conclusion, our results demonstrate that partial Mn deficiency is an additional key feature of *slc39a14* deficiency in zebrafish which should be considered in the treatment of affected individuals with SLC39A14 mutations. The *slc39a14* \(^{U801/−}\) loss-of-function zebrafish mutant is proving an invaluable disease model to study the disease pathogenesis of HMNDYT2 as well as Mn neurotoxicity in general.

Conflict of interest statement:

The authors declare no competing financial interests.

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References


Fordahl SC, Erikson KM (2014) Manganese accumulation in membrane fractions of primary astrocytes is associated with decreased gamma-aminobutyric acid (GABA) uptake, and is exacerbated by oleic acid and palmitate. Environ Toxicol Pharmacol 37:1148-1156.


Zofkova I, Davis M, Blahos J (2017) Trace elements have beneficial, as well as detrimental effects on bone homeostasis. Physiol Res 66:391-402.