Jessica Baesler | Vivien Michaelis | Michael Stiboller | Hajo Haase | Michael Aschner | Tanja Schwerdtle | Stephen R. Sturzenbaum | Julia Bornhorst

Nutritive Manganese and Zinc Overdosing in Aging C. elegans Result in a Metallothionein-Mediated Alteration in Metal Homeostasis

Suggested citation referring to the original publication:
Molecular Nutrition and Food Research 65 (2021) 8, Art. 2001176 pp. 1 - 11
DOI https://doi.org/https://doi.org/10.1002/mnfr.202001176
ISSN 1613-4133, 1613-4125

Journal article | Version of record

Secondary publication archived on the Publication Server of the University of Potsdam:
Zweitveröffentlichungen der Universität Potsdam : Mathematisch-Naturwissenschaftliche Reihe 1364
ISSN: 1866-8372
https://nbn-resolving.org/urn:nbn:de:kobv:517-opus4-514995
DOI: https://doi.org/10.25932/publishup-51499

Molecular Nutrition & Food Research
www.mnf-journal.com

Nutritive Manganese and Zinc Overdosing in Aging *C. elegans* Result in a Metallothionein-Mediated Alteration in Metal Homeostasis

*Jessica Baesler, Vivien Michaelis, Michael Stiboller, Hajo Haase, Michael Aschner, Tanja Schwerdtle, Stephen R. Sturzenbaum, and Julia Bornhorst**

Scope: Manganese (Mn) and zinc (Zn) are not only essential trace elements, but also potential exogenous risk factors for various diseases. Since the disturbed homeostasis of single metals can result in detrimental health effects, concerns have emerged regarding the consequences of excessive exposures to multiple metals, either via nutritional supplementation or parenteral nutrition. This study focuses on Mn-Zn-interactions in the nematode *Caenorhabditis elegans* (*C. elegans*) model, taking into account aspects related to aging and age-dependent neurodegeneration.
Methods and Results: Chronic co-exposure of *C. elegans* to Mn and Zn increases metal uptake, exceeding levels of single metal exposures. Supplementation with Mn and/or Zn also leads to an age-dependent increase in metal content, a decline in overall mRNA expression, and metal co-supplementation induced expression of target genes involved in Mn and Zn homeostasis, in particular metallothionein 1 (*mtl-1*). Studies in transgenic worms reveal that *mtl-1* played a prominent role in mediating age- and diet-dependent alterations in metal homeostasis. Metal dyshomeostasis is further induced in parkin-deficient nematodes (Parkinson's disease (PD) model), but this did not accelerate the age-dependent dopaminergic neurodegeneration.
Conclusions: A nutritive overdose of Mn and Zn can alter interactions between essential metals in an aging organism, and metallothionein 1 acts as a potential protective modulator in regulating homeostasis.

1. Introduction

Within the next 30 years, the share of elderly people (aged 65 years and older) is expected to increase to 16% of the world's population.[1] Concomitantly, the incidence of cardiovascular and neurodegenerative diseases, cancer and other pathological disorders will drastically increase, since aging is the primary risk factor for these disorders.[2] Studying to what extent nutrition, as an exogenous factor, contributes towards sustaining healthy aging and limiting the progression of age-related diseases is therefore timely.

Trace elements, such as zinc (Zn) and manganese (Mn) play essential roles in enzymatic reactions and signaling pathways, immune response or as structural components, which, in turn, also influence aging.[3] Organisms rely on tightly regulated homeostatic mechanisms, which are sustained by various transport and binding proteins, as well as stress response pathways.[4,5] Nutrition, the major route of essential trace metal intake, may cause an imbalance in these

J. Baesler, V. Michaelis, Prof. J. Bornhorst
Food Chemistry, Faculty of Mathematics and Natural Sciences
University of Wuppertal
Wuppertal, Germany
E-mail: bornhorst@uni-wuppertal.de
J. Baesler, Dr. M. Stiboller, Prof. T. Schwerdtle
Department of Food Chemistry, Institute of Nutritional Science
University of Potsdam
Nuthetal, Germany

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202001176

DOI: 10.1002/mnfr.202001176

J. Baesler, Prof. H. Haase, Prof. T. Schwerdtle, Prof. J. Bornhorst
TraceAge – DFG Research Unit FOR 2558
Berlin-Potsdam-Jena, Germany
Prof. H. Haase
Department of Food Chemistry and Toxicology
TU Berlin
Berlin, Germany
Prof. M. Aschner
Department of Molecular Pharmacology, Neuroscience, and Pediatrics
Albert Einstein College of Medicine
NY, USA
Prof. M. Aschner
IM Sechenov First Moscow State Medical University
Moscow, Russia
Prof. S. R. Sturzenbaum
Department of Analytical, Environmental & Forensic Sciences, School of Population Health & Environmental Sciences, Faculty of Life Sciences & Medicine
King's College London
London, UK

complex networks, potentially contributing to the progression of aging and age-related disorders.

In 2013, the European Food Safety Authority (EFSA) set an Adequate Intake (AI) for Mn in adults at 3 mg·day^{-1}.[6] This AI can be exceeded by drinking Mn-adulterated water[7] or long-term parenteral nutrition (PN), to name a few.[8] Chronic overexposure to Mn is known to cause neurotoxicity, referred to as manganism, a parkinsonism-like disorder.[9]

Severe outcomes of Zn dyshomeostasis are largely due to malnutrition, which is especially prevalent in the elderly population. Vural et al., for example, reported that an insufficient daily Zn intake (defined as below the estimated average requirement (EAR) of 6.8 mg Zn·day^{-1} for women and 9.4 mg Zn·day^{-1} for men) was observed in 31–66% of older adults.[10] Zn supplements have been shown to positively affect the immune system, but chronic overexposure may exert negative effects on immune function, impair metal homeostasis, and induce apoptosis.[11] Thus, excessive administration of Zn and/or Mn may represent a risk factor for degenerative disorders, such as Parkinson's disease (PD).[12,13]

Mixtures of trace elements can alter environmental availability, as well as their toxicokinetic and toxicodynamic properties;[14] however specifics pertaining to their competition during absorption, distribution and cellular uptake, as well as their interactions are rarely characterized. Mn and iron (Fe) compete for uptake via the divalent metal transporter 1 (DMT1), ferroportin (FPN) and the transferrin (Tf)/transferrin receptor (TfR) machinery, which can result in dysregulation of metal homeostasis.[15,16] Zn can affect Fe homeostasis, especially during Fe deficiency, by increasing DMT1 and FPN1 expression, or by decreasing the levels of hepcidin.[17] Mn and Zn also share several transport mechanisms, including DMT1,[18] as well as the cation-transporting lysosomal p-type ATPase ATP13A2.[4,19] Likewise, the Zn transporters ZIP8 and ZIP14 regulate hepatic, alveolar and renal Mn (re)uptake,[16] and FPN is induced by Zn and acts as a Mn transporter.[17] Taken together, a plethora of data suggest a regulatory relationship between Mn and Zn; however the effects of concomitant exposure to multiple metals have, at large, yet to be explored.

Accordingly, the present study aimed to outline the underlying effects of a chronic nutritive Mn and Zn overexposure on metal homeostasis and neurodegeneration during the aging process in the nematode *Caenorhabditis elegans (C. elegans)*, a powerful invertebrate model for aging and neurodegeneration research.[20] We aimed to extend the current understanding of healthy and neuronal aging by identifying and characterizing the evolutionary conserved drivers of tightly regulated homeostatic networks.

2. Results

2.1. Mn and Zn Bioavailability in Aging Wildtype

The chosen concentrations of Zn and/or Mn did not affect overall lifespan, suggesting that the chronic feeding regime with metal enriched *Escherichia coli (E. coli)* did not affect *C. elegans* lifespan (**Figure 1**A). The concentrations following Mn and Zn supplementation exceeded the physiological need to represent a chronic overexposure which is able to induce dyshomeostases. The median lifespan was 12 days (after L4) (Figure 1A), and this time point was chosen as the experimental "late-life" condition. Young (fecund state) and middle-aged worms (end of reproductive period exhibiting first aging characteristics) were defined as 2 days or 5 days post L4, respectively.

The basal trace element status (Mn, Fe, Cu and Zn) of aging worms (Figure 1B) showed an age-dependent increase in Fe levels, and a tendency towards an enrichment of Mn and Zn. Nutritive Mn overexposure during the aging process led to a time-dependent increase in Mn in wildtype worms (Figure 1C). Moreover, the combined supplementation of Mn with Zn further increased Mn concentrations by about two-fold in middle-aged (day 5 post L4) and old (day 12 post L4) worms. Zn levels increased in young and middle-aged wildtype *C. elegans*, but not significantly in old worms (Figure 1D). The combined supplementation of 5 mM Mn and 1 mM Zn increased Zn levels compared to their respective controls at all tested life stages, and also increased Zn levels in young and middle-aged worms when compared to high-dose single metal Zn supplementation. This effect was also observed in worms challenged for 5 days with 5 mM Mn and 0.5 mM Zn, where Zn levels increased by 6.3-fold compared to worms exposed solely to 0.5 mM Zn.

2.2. Transcriptional Regulation of Metal Homeostasis Upon Chronic Mn and Zn Supplementation

Transcripts previously identified as key players in the homeostasis of Mn and Zn, were chosen to assess their response within the context of combined metal exposure in aging worms. qPCR analysis of *smf-3*, which encodes the main Mn importer in *C. elegans* and is orthologous to the mammalian DMT-1,[25] was induced in young worms exposed to Mn, in the presence or absence of Zn supplementation (**Figure 2**A). This effect was observed in aged worms co-exposed to Mn and Zn, but notably not in worms exposed to a single metal (Figure 2A). Gene expression of *fpn-1.1* (the main Mn exporter in *C. elegans* and the orthologue of mammalian FPN[26]) was indistinguishable between Mn supplementation only and the combined metal-enriched diets (Figure 2B). mRNA levels of the cytosolic Zn sequester *cdf-2* (the orthologue of mammalian ZnT2)[27] was induced by a diet supplemented with Zn and Mn, but was not affected by single Zn or Mn supplementation (**Figure 3**C). Changes in expression of *ttm-1*, which is related to the mammalian zinc transporters of the cation diffusion facilitator (CDF) family,[28] were less marked. In young and late-life worms the combined supplementation with Mn and Zn induced *ttm-1* gene expression (Figure 2D). *C. elegans* expresses metallothioneins, *mtl-1* and *mtl-2*, although both are involved in metal metabolism, they are thought to exhibit differential metal binding preferences.[29,30] *Mtl-1* expression was strongly up-regulated in young and middle-aged worms co-exposed to Mn and Zn (Figure 2E). In contrast, we observed only minor changes in *mtl-2* expression levels with most changes limited to young worms co-exposed to 5 mM Mn and 1 mM Zn (Figure 2F).

2.3. Alterations of Metal Homeostasis in *mtl-1* Deletion Mutants

Since Mn and Zn co-exposure resulted in a substantial up-regulation of *mtl-1* mRNA expression in *C. elegans* wildtype, the effects on Mn and Zn homeostasis in *mtl-1* knockout mutants

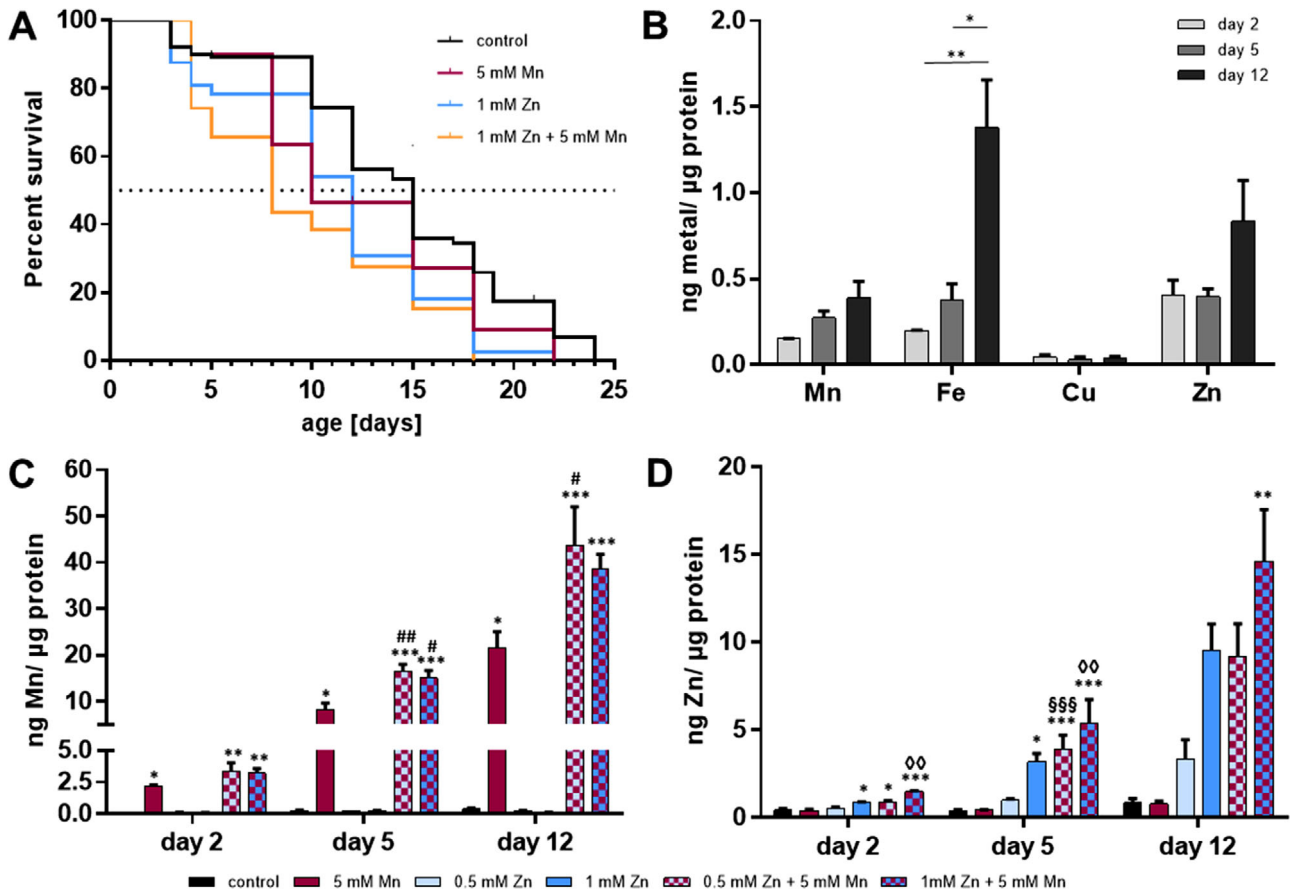

Figure 1. Mn and Zn homeostasis in aging wildtype (WT) worms. A) Kaplan-Meier survival curves of WT chronically fed with metal enriched *E. coli* during adulthood. EC50/median survival plotted as dotted line. B) Total metal content in young (day 2), middle-aged (day 5) and late-life (day 12) WT adults. */** indicate age-related changes identified by one-way ANOVA. C) Mn and D) Zn bioavailability in young (day 2), middle-aged (day 5) and late-life stage (day 12) WT adults upon chronic Mn and/or Zn supplementation with respective metal concentrations during adulthood with one-way ANOVA statistics indicated as follows: *, #, ∫ and ◇: compared to control, 5 mM Mn, 0.5 mM Zn, or 1 mM Zn values of the respective same age. Values of B-D were determined by ICP-MS/MS, normalized to protein content and expressed as means + SEM of 3 replicates.

(tm1770) were explored further, in order to elucidate MTL-1's role in regulating the overexposure to metals.

Since *mtl-1* deletion mutants exhibited a reduced lifespan phenotype (median lifespan of 8.5 days) compared to wildtype worms (Figure 1A), analyses were carried out only in young (day 2) and middle-aged worms (day 5) (Figure 3A). Feeding a Mn-supplemented diet to *mtl-1* mutants until day 2 and day 5 of adulthood caused an increase in Mn compared to their respective controls (Figure 3B), with values comparable to those in wildtype worms. However, combined exposure to Mn and Zn did not alter Mn levels above those inherent to worms exposed to Mn alone (as seen with wildtype worms). Interestingly, Mn concentrations in mutants fed with *E. coli* only or Zn-enriched diets was typically lower (about 10-fold in day 5 adults) than the values obtained from their respective wildtype counterparts (statistical parameters of strain comparisons are summarized in supplementary table S4). An increase in Zn levels in day 2 adults was only observed in worms fed with 1 mM Zn or the combination of 1 mM Zn and 5 mM Mn (Figure 3C). Exposure of *mtl-1*-mutant worms to the metal mixtures resulted in accumulation of Zn at day 5, however, their values were significantly lower than those in wildtype worms of similar age and dosing-protocol, namely -54% (5 mM Mn + 0.5 mM Zn) and -44% (5 mM Mn + 1 mM Zn) (see Figure 3C and Table S4).

To better understand the interplay between drivers of homeostatic regulation, further transcripts were assessed in the *mtl-1* mutant. The *mtl-2* mRNA levels were generally about 10-fold higher in the *mtl-1* transgenic worms than the corresponding values in wildtype worms (see Figure 3D, 2F and Table S4). In addition, *mtl-2* gene expression in the mutants typically increased significantly upon supplementation with the combined metals. The expression of *cdf-2* in *mtl-1*-deficient worms was induced by high Zn as well as Mn and Zn co-exposures in day 2 adults, but only in day 5 adults grown on plates containing 1 mM Zn and 5 mM Mn (Figure 3E). However, the strain comparison revealed that Zn increased *cdf-2* expression in the *mtl-1* mutant to a significantly higher extent than in wildtype worms (Table S4). Furthermore, aging effects emerged, since the expression of *smf-3* was significantly higher in day 2 and day 5 adults in *mtl-1* mutants compared to wildtype, and similarly, *fpn-1.1* mRNA levels increased in middle-aged *mtl-1* mutants (see supplementary Figure S5 and Table S4).

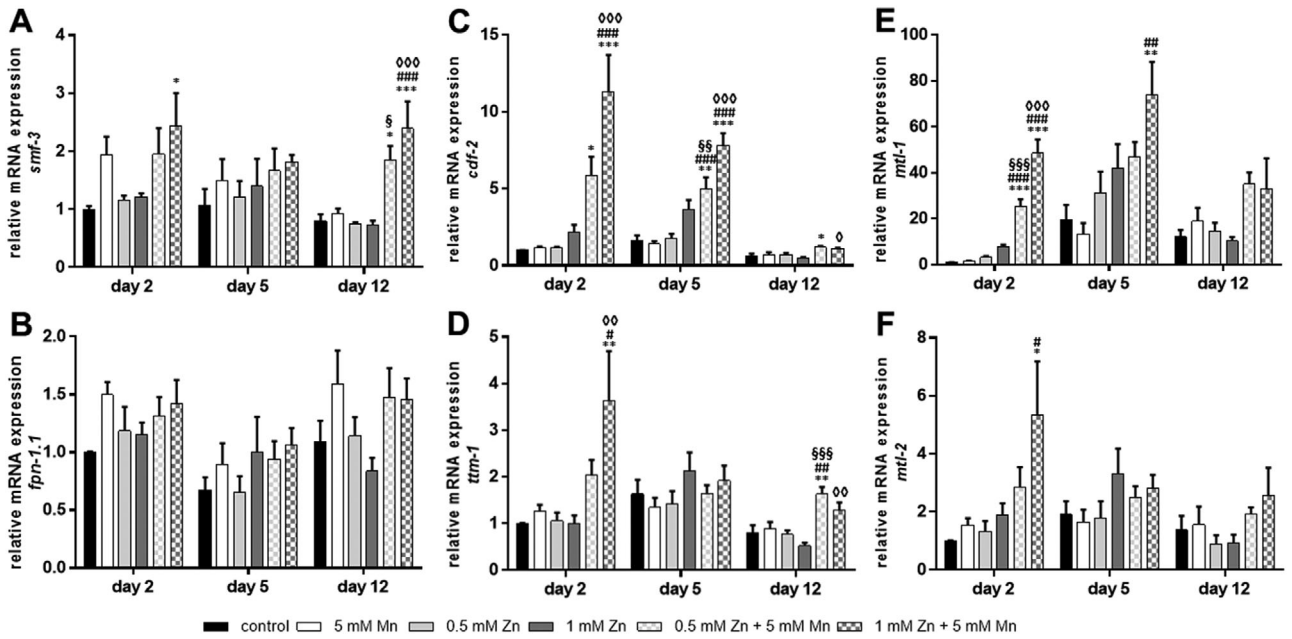

Figure 2. Relative mRNA expression of metal homeostasis-associated genes in aging wildtype worms (day 2, 5 and 12 of adulthood) following chronic Mn and/or Zn supplementation during adulthood. Relative gene expression was determined by RT-qPCR analysis and normalized to β-actin homologue *afd-1*. Data are expressed as means + SEM of three biological replicates relative to day 2 controls. One-way ANOVA statistics are indicated as follows: *, #, § and ◊: compared to control, 5 mM Mn, 0.5 mM Zn, or 1 mM Zn values of the respective same age.

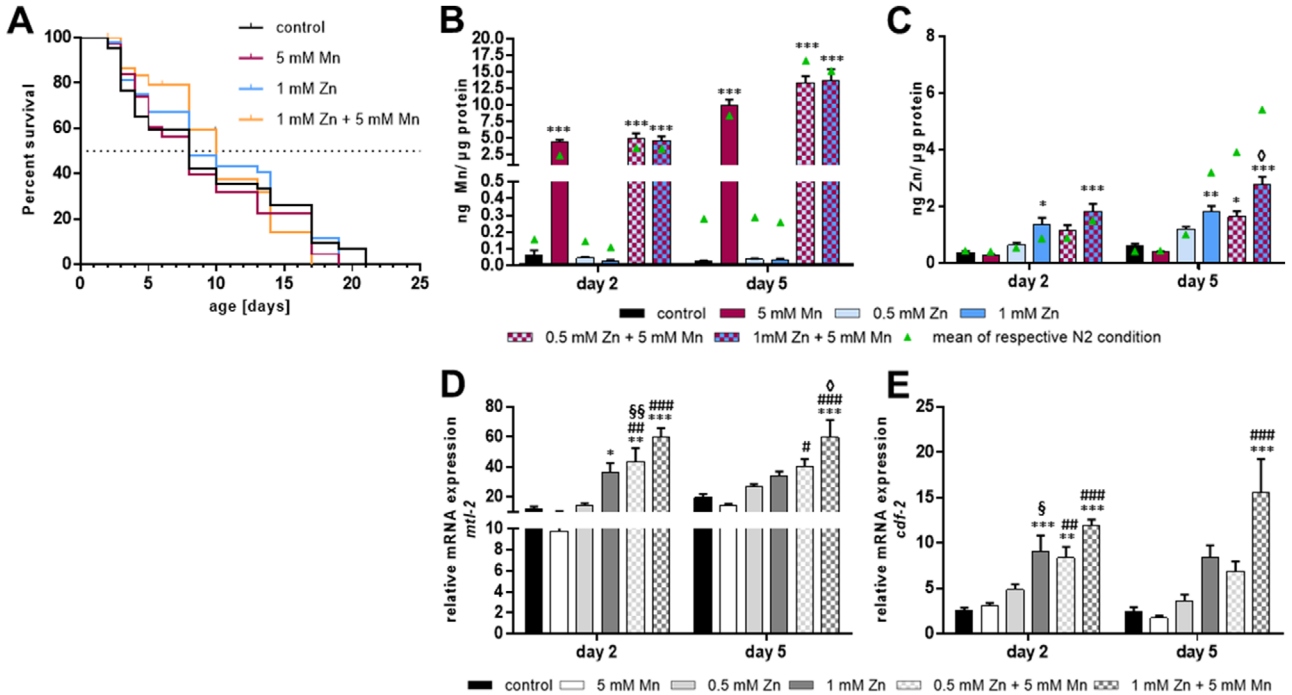

Figure 3. Mn and Zn homeostasis and related gene expression in *mtl-1*-deficient mutant worms. A) Kaplan Meier survival curves of tm1770 chronically fed with metal enriched *E. coli* during adulthood. EC50/median survival plotted as dotted line. B) Mn and C) Zn bioavailability of young (day 2), middle-aged (day 5) and late-life stage (day 12) tm1770 adults upon chronic Mn and/or Zn supplementation during adulthood as determined by ICP-MS/MS analysis. D) *Mtl-2* and E) *cdf-2* mRNA expression relative to WT day 2 controls, as measured via RT-qPCR and normalized to β-actin homologue *afd-1*. Data of B-E are expressed as means + SEM of three biological replicates each with one-way ANOVA statistics indicated as follows: *, #, § and ◊: compared to control, 5 mM Mn, 0.5 mM Zn, or 1 mM Zn values of the respective same age.

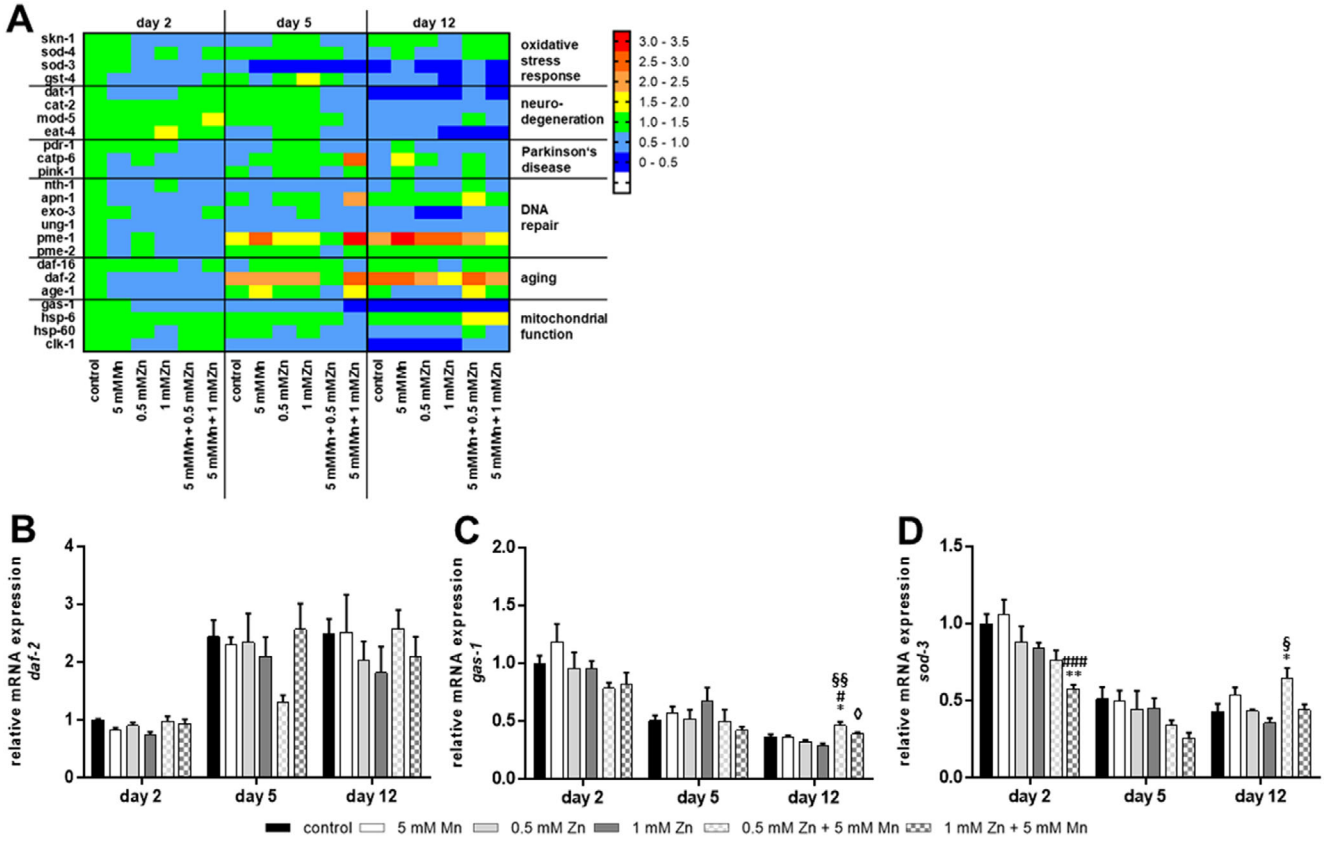

Figure 4. Relative mRNA expression of toxicologically relevant genes in aging wildtype worms (day 2, 5 and 12 of adulthood) following chronic Mn and/or Zn supplementation during adulthood. Relative gene expression was determined by RT-qPCR analysis and normalized to β-actin homologue *afd-1*. Data of heat map (A) and graphs B-D are expressed as means or means + SEM of three biological replicates relative to day 2 controls. One-way ANOVA statistics in B-D are indicated as follows: *, #, § and ◊: compared to control, 5 mM Mn, 0.5 mM Zn, or 1 mM Zn values of the respective same age.

2.4. Influence of Metal Supplementation and Aging on Toxicity-Related Pathways

Oxidative stress responses, neurodegeneration, DNA repair and mitochondrial function have been linked to Mn toxicity, Zn toxicity, as well as aging.[31,13,32] In order to evaluate the consequences of overdosing Mn and/or Zn in greater depth, changes in 24 transcripts within these pathways were investigated as summarized in **Figure 4**A. The majority of expression levels was not affected by metal supplementation, but several aging effects were observed, the most striking ones depicted in Figure 4B–D. *Daf-2* expression, the orthologue of mammalian insulin/insulin-like growth factor 1 (IGF1) receptor and upstream target of the insulin/IGF1 signaling cascade (IIS),[33] was age-dependently increased by a factor of 2 (Figure 4B). In contrast, mRNA level of *gas-1*, which encodes for a component of complex I in the mitochondrial respiratory electron chain,[34] was reduced by 50% in middle-aged and late-life worms (Figure 4C). Interestingly, day 12 adults exhibited an increase in *gas-1* expression when co-exposed to Mn and Zn. Likewise, expression of the Mn-dependent superoxide dismutase *sod-3* decreased in an age-dependent manner (Figure 4D).

2.5. Influence of Metal Supplementation and Aging on Dopaminergic Neurodegeneration

Neurodegeneration is a key outcome of Mn and Zn toxicity as well as aging. Accordingly, we investigated outcomes of neuronal damage upon chronic metal supplementation. As shown in **Figure 5**A, *dat-1* expression declined age-dependently in wildtype worms at day 12 of adulthood. A similar decrease at this late-life stage was also noted for *cat-2* and *eat-4*, which encode for tyrosine hydroxylase and a vesicular glutamate transporter, respectively (Figure 4A, not shown in detail). Furthermore, damage to cephalic dopaminergic neurons in the form of occurrence of discontinuous or lost dendrites, dendritic blebbing and shrunken, splinted or lost soma was quantified in BY200 worms, which express the Pdat-1::GFP transgene (examples depicted in Figure 5C). While an age-dependent increase in neurodegenerative events was observed, dietary metal exposure had only minor effects on neuronal health (Figure 5B). Generally, the fluorescence signal of *dat-1* was indistinguishable between the different supplementation groups, but consistently became weaker and blurred with increased age (Figure 5D, not all conditions shown).

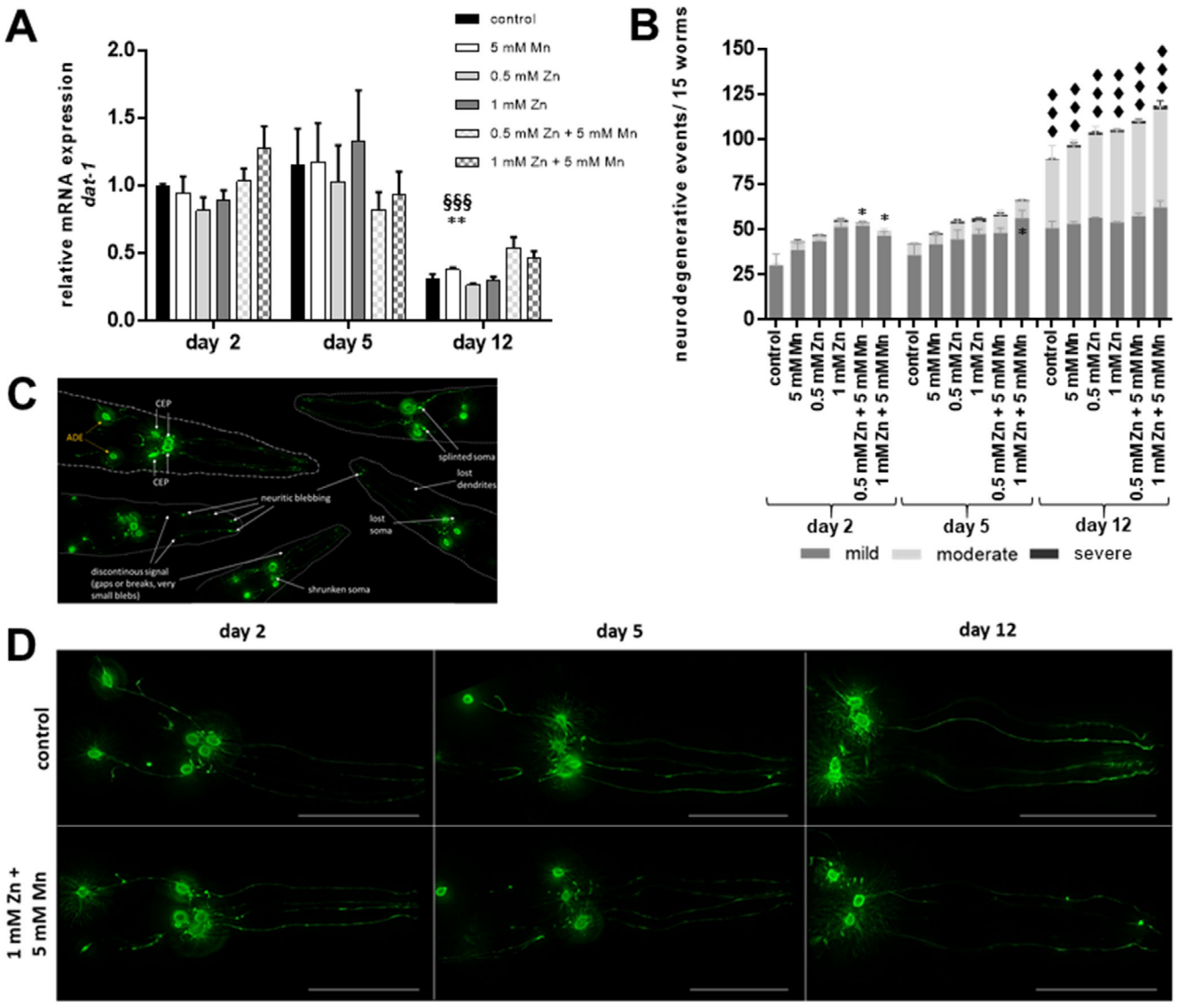

Figure 5. Dopaminergic neurodegeneration in aging wildtype worms (day 2, 5 and 12 of adulthood) following chronic Mn and/or Zn supplementation during adulthood. A) Relative mRNA expression of *dat-1* relative to day 2 controls determined by RT-qPCR analysis of three biological replicates normalized to *β*-actin homologue *afd-1* and expressed as means + SEM with one-way ANOVA statistics indicated as follows: *, # and §: compared to control, 5 mM Mn or 0.5 mM Zn values of the respective same age. B) Neurodegeneration of dopaminergic neurons in BY200. CEP neurons of 15 transgenic worms with GFP-tagged DAT-1 were categorized according to mild, moderate, or severe damages of neurons using fluorescence microscopy. Data are expressed as means + SEM from three replicates. One-way ANOVA statistics are indicated as follows: *: compared to control of the respective same age and ♦: compared to day 2 and day 5 of the respective same condition. C) Representative fluorescence images of typical neurodegenerative events of dopaminergic neurons. D) Representative fluorescence images of BY200 dopaminergic neurons following chronical feeding of control or metal enriched (1 mM Zn + 5 mM Mn) *E. coli* during adulthood. Scale bars represent 50 µm.

2.6. Homeostatic and Neurodegenerative Effects of Mn and Zn Supplementation on *pdr-1*-deficient Worms Resembling a PD Model

Dopaminergic neurodegeneration is a known endpoint of chronic metal exposure in PD, and Mn has been established as a causative metal in the etiology of this disease.[35] Here, we examined effects of a chronic metal supplementation on PD mutants deficient for the E3 ubiquitin ligase parkin, which is encoded by *pdr-1*.[36]

ICP-MS/MS analysis of *pdr-1* mutants revealed increased Mn levels upon chronic supplementation with Mn or Mn and Zn combined at all age stages (**Figure 6**A). However, compared to wildtype worms, the loss of *pdr-1* resulted in decreased Mn levels in late-life stage worms fed with Mn or Mn and Zn enriched *E. coli*. The bioavailability of Mn in day 12 adult wildtype worms was approximately twice as high (compare with Figure 1C). The accumulation of Mn was less pronounced in late-life *pdr-1* mutants compared to wildtype worms exposed to Mn and Zn. Zn levels in middle-aged and late-life worms remained, at large,

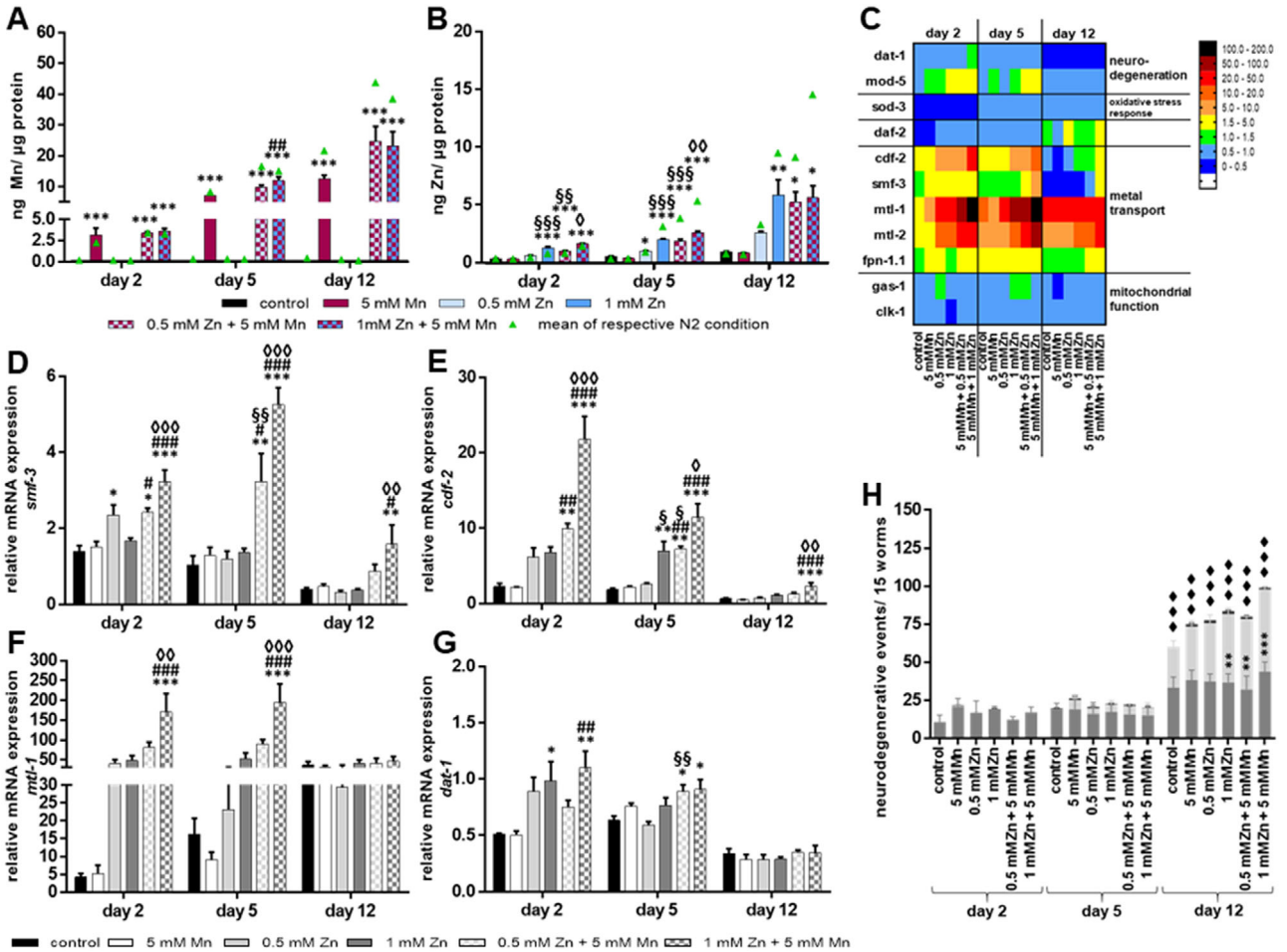

Figure 6. Mn and Zn homeostasis, gene expression, and neurodegeneration of young (day 2), middle-aged (day 5), and late-life stage (day 12) *pdr-1*-deficient PD mutants (VC1024) following chronic Mn and/or Zn supplementation of indicated concentrations during adulthood. A) Mn and B) Zn bioavailability as determined by ICP-MS/MS analysis. C-G) mRNA expression of genes related to metal homeostasis and toxicity relative to WT day 2 controls, as measured via RT-qPCR and normalized to *β*-actin homologue *afd-1*. H) Neurodegeneration of dopaminergic neurons in MAB8. CEP neurons of 15 transgenic worms with GFP-tagged DAT-1 each were categorized according to mild, moderate or severe damages to neurons using fluorescence microscopy. Data of A-B and D-H are expressed as means + SEM of three biological replicates each with one-way ANOVA statistics indicated as follows: *, #, §, and ◇: compared to control, 5 mM Mn, 0.5 mM Zn, or 1 mM Zn values for the respective same age and ♦: compared to day 2 and day 5 of the respective same condition. Data in C depict mean values of three replicate measurements.

lower than in wildtype worms (Figure 6B). However, Mn and Zn co-supplementation resulted in increased Zn levels in young and middle-aged worms, when compared to Zn-only exposures.

Interestingly, the expression of *smf-3* and *cdf-2* in *pdr-1* mutants was higher upon co-supplementation of Mn and Zn (compared to single metal exposures) in all tested life stages (with the exception of day 12 adults exposed to 0.5 mM Zn and 5 mM Mn) (Figure 6C–E). In addition, the increase in *smf-3* expression at day 5, as well as the expression of *cdf-2* in young and middle-aged worms was markedly higher when compared to wildtype worms (Figure 2A,C, supplementary Table S4). The expression of both genes declined at late-life stage in the *pdr-1* mutant. In addition, *mtl-1* expression was also found to be up-regulated in the *pdr-1* mutant in an age-dependent manner, even in the absence of metal supplementation. The expression of *mtl-1* was 3-fold higher in young and middle-aged *pdr-1* mutant worms exposed to 1 mM Zn and 5 mM Mn, compared to their wildtype counterparts (Figure 6F).

Finally, we investigated dopaminergic neurodegeneration in *pdr-1*-deficient *C. elegans*, since it is one of the key characteristics of PD and manganism. As depicted in Figure 6G, the basic levels of *dat-1* expression were lower in young *pdr-1* mutants, when compared to wildtype worms. In addition, *dat-1* expression in young and middle-aged *pdr-1* mutants was partly induced by 1 mM Zn or the metal co-exposure. Figure 6H shows the age-dependent increase of degenerated neurons in *pdr-1*-deficient mutants. In general, the neurons of the *pdr-1* mutants seemed less degenerated than those observed in BY200 (Figure 5D and supplementary Figure S6). However, late-life stage *pdr-1* mutants fed with 1 mM Zn or Mn and Zn enriched diets revealed moderate degenerative changes when compared to controls (Figure 6H).

3. Discussion

Essential trace elements, such as Mn and Zn, are essential for a plethora of physiological functions associated with aging. However, little is known about the molecular mechanisms of trace element interactions, and to what extent their profiles change during aging. Our study corroborated previous results regarding the age-dependent accumulation of Fe and Zn in worms.[37–39,22] Regarding Cu homeostasis, our findings failed to reveal increased levels in this trace metal as a function of age, as previously reported by Klang et al. and Sauzéat et al., which might be due to differences in study design, such as worm propagation methods.[37,38] A comparison of the observed age-specific trace element alterations in worms with those encountered in body fluids (i.e., serum/plasma) of aging patients is challenging, requiring additional data.[40]

Given that metal exposure can impair early development,[39] we opted to start the supplementation at the late L4 larvae stage with continuation throughout the adult stage. In wildtype worms, the single metal supplementation resulted in an increase of Mn or Zn levels over the exposure time. Mn supplementation did not increase Zn levels, as observed in another study where worms were raised for 24 h on Mn-enriched agar.[41] Kumar et al. reported increased Zn levels, but no effect on Mn bioavailability in young worms supplemented with Zn,[39] corroborating our findings. Compared to single metal supplementation, chronic co-exposure to Mn or Zn with the respective other trace element further increased their bioavailability, especially during aging, indicating the presence of synergistic homeostatic mechanisms for metal regulation. Although comparative data for *C. elegans* are lacking, studies in other organisms partly corroborate our findings. For example Mn-exposure resulted in elevated Mn and Zn levels in brains of Sprague-Dawley rat pups, and a co-incubation of Mn and Zn was found to increase Mn levels and alter subcellular distribution of Mn in primary chick glial cells.[42,18] Furthermore, Mercandate et al. noted that the route of exposure may play an important role for the homeostatic interaction of metals, since Zn accumulated in male Sprague-Dawley rats chronically exposed by gavage to Mn, but not when introduced via dietary supplementation.[43] However, other studies were not able to pinpoint changes in the Zn levels of rat brains upon Mn exposure.[44] This highlights the current controversy regarding the impact of interacting factors within the field of trace element homeostasis.

The homeostasis of trace elements is crucial for maintaining biological functions and preventing metal-induced health issues. Thus, the potential competition in transport of Mn and Zn as well as homeostasis-related proteins need to be considered, especially in the context of co-exposure and aging. At the transcriptional level, analysis of the most important regulators of Mn and Zn homeostasis in aging *C. elegans* revealed only minor regulatory effects, although acute Mn exposure in L1 larvae decreased *smf-3* gene and protein expression.[25] Both, Mn and Zn did not affect gene expression of the FPN1 homologue *fpn-1.1* in wildtype worms, although FPN1 was previously shown to attenuate Mn and Zn toxicity in frogs and bone marrow macrophages of mice.[45,46] This could partly be due to different signaling, since Zn, (but not Fe) have been shown to induce ferroportin expression in macrophages dependent on the metal-activated transcription factor MTF1,[45] which notably is not expressed in *C. elegans*. Combined Mn and Zn supplementation significantly increased the expression of *cdf-2*, which encodes a zinc transporter of the cation diffusion facilitator family, highly homologous to human ZnT2 and *ttm-1*, a gene related to human Zn transporters ZnT2-8 and predicted to be involved in Zn excretion by transporting Zn into the intestinal lumen.[27,28] CDF-2 is expressed in intestinal cells and transports Zn from the cytosol into lysosome-related gut granules.[47] The implied enhanced metal detoxification was supported by the substantial increase in *mtl-1* expression in young and middle-aged wildtype worms co-supplemented with Mn and Zn, but not Zn alone. *Mtl-1* is known to be involved in metal detoxification, but is thought to have a preference for Zn binding, thereby preventing a hyperaccumulation of Zn.[29,30,48] *C. elegans mtl-1* and *mtl-2* seem to exhibit distinct roles, and this is supported by observation that whilst *mtl-1* expression was highly responsive to Mn and Zn supplementation, *mtl-2* expression was less affected.[29,30] The expression of *mtl-1* is affected by age[49] consistent with our observations that *mtl-1* increased until day 5 of adulthood, with its levels declining thereafter, especially in *C. elegans* challenged with Mn and Zn. The results of *mtl-1* deletion mutants obtained in this study demonstrate that *mtl-1* plays a crucial role in enhancing the bioavailability of Mn and Zn when administered simultaneously. Although not via co-exposure, a relationship between these two metals and metallothioneins was previously found in mice, as Mn treatment caused interleukin-6 and ZIP14 induction in the liver, resulting in induced levels of hepatic Zn metallothionein.[50]

Mn and Zn play essential roles in apoptosis, oxidative stress responses and mitochondrial function. The maintenance of their physiological function is highly dependent on tightly regulated homeostasis. The present study confirmed aging effects in some of these pathways. For example, the *gas-1* gene, which encodes an integral protein of complex I of the mitochondrial electron transport chain,[34] was age-dependently decreased, supporting the theory that mitochondrial dysfunction acts as one of the hallmarks of aging.[32] The expression of *daf-2* was, however, not age-dependently altered. Likewise, no significant changes in expression of other typical IIS mediators such as *skn-1*, *daf-16*, or *age-1* were observed, suggesting that within the context of this study IIS may not be the main driver responsible for compensating metal-induced stress. The Mn-dependent superoxide dismutase *sod-3* decreased as worms aged, as well as in young worms co-exposed to Mn and Zn, which broadly corroborates previous reports,[51] but contradicts Schetinger et al. who found increased *sod-3* expression in worms co-exposed to Mn and mercury (Hg), but not to the respective single metals.[52] However, it should be noted that the applied exposure methods differed, namely Schetinger et al. investigated the effect of acute exposures to L1 larvae and measured gene expression 24 h post treatment. Therefore, *sod-3* might serve as an acute antioxidative response mechanism and demonstrates that the route and duration of metal exposure may alter the acute and adaptive responses to exogenous stressors. Whilst antibodies specific for the respective *C. elegans* proteins are currently not available, they could, in the future, be used to establish the correlation between mRNA and protein expression.

Various mechanisms such as oxidative stress, protein aggregation, mitochondrial dysfunction or energy deficiency have

linked excessive Mn exposure and resulting dyshomeostasis to the pathogenesis of neurodegenerative disorders, e.g. by causing dopaminergic cell death.[53] The role of Zn remains controversially discussed in this context, but some studies suggested intracellular Zn accumulates in in vitro and in vivo PD models, accompanied by dopaminergic cell death.[54] A decrease in dopamine levels and the loss of dopamine neurons are hallmarks of human PD brains.[55] Here, aging affected the expression of the dopamine reuptake transporter gene *dat-1*, and the number of degenerated cephalic dopaminergic neurons (CEPs) significantly increased in wildtype worms of late-life stage. But metal exposure did not alter the expression of *dat-1*, nor did it induce damage to dopaminergic neurons, which contradicts the Mn dose- and time-dependent induction of neuronal damage reported by others.[35] The effect on neurodegeneration was assessed in *pdr-1* mutants, worms lacking the parkin orthologue, an E2-dependent E3 ubiquitin ligase that promotes autophagy of mitochondria by proteasomal degradation.[56] In general, the *pdr-1* mutants' response to Mn and/or Zn supplementation was similar to wildtype worms, but the number of degenerative events increased more drastically in late-life worms. The results corroborate findings by Cooper *et al*. who claimed that the loss of *pdr-1* fails to cause loss or death of dopaminergic neurons.[57] Compared to wildtype, the PD model exhibits altered bioavailability of Zn and Mn, with a trend towards diminished metal load at late-life stage and changed expression patterns of homeostasis-associated genes upon chronic co-exposure to Mn and Zn, as reflected by the massive increase in *mtl-1* expression. This implicates a link between the induction of detoxifying metal transporters and impaired proteasomal degradation of mitochondria. In a recent study Pretsch et al. have shown the age-dependent potential of supplemented Zn to increase and prolong metallothionein induction and thereby decrease proteotoxicity in amyloid β and α-synuclein expressing worms.[58] Taken together, this indicates that the induction of metal dyshomeostasis seems to be alleviated during aging and in terms of PD due to altered regulation mechanisms, in turn, rendering organisms more susceptible to the toxicity of metal mixtures.

In summary, this study revealed that chronic nutritive co-exposure to the essential metals, Zn and Mn, in *C. elegans* augmented their uptake and the expression of target genes. Furthermore, the metal-chelating molecule, metallothionein 1, seems to play a prominent role in the mediation of age- and diet-dependent alterations of Zn and Mn homeostasis.

Mn and Zn are typically added as micronutrients to food, supplements or PN formulas, and therefore their effects should not be studied in isolation, but rather considered in concert, in particular in the context of aged organisms. The route and length of application seems to play a critical role in this context, since chronic co-supplementation of Mn and Zn alters both their bioavailability and their homeostatic regulation. Chronic supplementation of Mn and Zn seems to affect metal homeostasis to a greater extent than acute intake, but, in turn, facilitates the activation of adaptive mechanisms. Taken together, our novel results highlight the need to further study the interplay of metals more closely, as their effects are likely to be synergistic in nature and thus represent critical underpinnings in the etiology of neurodegenerative diseases. Furthermore, future research might explore whether patients with elevated Mn and Zn levels (for example in brain tissue) are characterized by increased expression of genes that regulate metal homeostases.

4. Experimental Section

C. elegans Strains and Maintenance: Mixed populations of *C. elegans* were grown on 8P agar plates covered with NA22 *E. coli* according to published protocols.[21] Eggs were isolated following sodium hypochlorite treatment (1% NaOCl and 0.25 M NaOH) and allowed to develop at 20 °C to age-synchronous L4 populations on Nematode Growth Media (NGM) plates containing OP50 as a bacterial food source.

Strains: N2 Bristol, *wildtype* and the deletion mutant VC1024 (*pdr1(gk448) III*) were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, Minneapolis, USA. The *mtl-1* deletion strain tm1770, MAB8 (*pdr-1(gk448) III; vtIs7[Pdat-1:GFP]*) and BY200 (*vtIs1 [Pdat-1::GFP; rol-6] V*, roller phenotype rarely detectable) were kindly provided by the Sturzenbaum laboratory (King's College, London, UK), the Aschner laboratory (Albert Einstein College of Medicine, NY, USA) and the Blakely laboratory (Vanderbilt University Medical Center, Nashville, USA).

Chronic Metal Supplementation: All solutions were prepared with purified water (10 MΩ•cm^{-1}) obtained from an Elix water purification system (Merck Millipore, Merck KGaA, Darmstadt, Germany). Stock solutions of 1 M $ZnSO_4$ ($ZnSO_4$•$7H_2O$, ≥ 99.5%, Merck KGaA) and 2 M $MnCl_2$ ($MnCl_2$·$4H_2O$, 99.99% trace metals basis, Honeywell, Morristown, USA) in 85 mM NaCl (>99.8%, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were freshly prepared weekly and stored at 4 °C.

To chronically expose the worms, inactivated OP50 solution (control), which normally contains 30.59 µM Zn and 0.91 µM Mn, was enriched with either 1 M $ZnSO_4$ and/or 2 M $MnCl_2$ to obtain concentrations of 5 mM Mn, 0.5 mM Zn, 1 mM Zn, or their respective combinations. Prior to experimentation, the OP50 solution was prepared by inoculating LB/Strep medium (25 mg·mL^{-1} LB Broth, 1 mg·mL^{-1} streptomycin sulfate, ≥ 720, I.E. mg^{-1}, Cellpure, both from Carl Roth GmbH + Co. KG) with OP50 bacteria (CGC) for 2 days at 37 °C and mild shaking. Bacterial growth was inhibited by heat-inactivation for 4 h at 70 °C in a water bath to prevent transformation of added metal species by the bacteria.

750 L4 larvae were placed onto control or metal-enriched OP50-covered NGM plates. To ensure age homogeneity as well as ad libitum food supply, adult worms were washed off the plates and separated (daily) from their offspring until they reached day 6 of adulthood, afterwards this was repeated every other day, as described elsewhere.[22] On days 2, 5and 12 of adulthood, worms were washed in 85 mM NaCl to remove offspring and bacterial residues.

Lifespan Assay: L4 larvae (20–30) were placed, in duplicates, on 3.5 cm NGM plates covered with concentrated (control) or metal enriched OP50 (see section above). Living worms were counted daily and transferred onto fresh plates with a platinum wire to separate them from offspring and dead worms. From day 6 of adulthood, worms were transferred to fresh plates every other to third day until no living worms remained. Worms were censored when they were stuck in the agar or showed signs of worm bagging or vulva ruptures. Worms were scored as dead when they failed to move after gentle prodding of their heads with fine wire.

Metal Quantification via ICP-MS/MS: To determine the total metal content (Mn, Zn, Fe, Cu) by inductively coupled plasma tandem mass spectrometry (ICP-MS/MS), pellets of 1500 *C. elegans* in 0.5 mL 85 mM NaCl were collected, flash frozen in liquid nitrogen and stored at -80 °C on days 2, 5 and 12 of adulthood.

Total metal concentrations were normalized to the protein content. Briefly, pellets were thawed, resuspended and sonicated (according to worms age and size 5–8 alternating cycles of 20 s sonication with 100% amplitude and 30 s cooling on ice to prevent denaturation). After centrifugation (4 °C, 5 min, 24 100 rcf) the protein content of the supernatant was determined with the bicinchoninic acid (BCA) assay (Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

Acid mineralization of pellets and metal analysis in digested samples were performed as previously described[22] with a MARS 6 microwave

system (CEM, Kamp-Lintfort, Germany) and an Agilent ICP-MS/MS (ICP-QQQ-MS 8800, Agilent, Waldbronn, Germany), respectively. ICP-MS/MS settings are provided in Table S1. The certified reference material ERM-BB422 (fish muscle) (Institute for Reference Materials and Measurement of the European Commission, Geel, Belgium) was used for quality control purposes. Measured concentrations for analyzed metals were in good agreement with certified concentrations of respective elements as shown in Table S2 (supplementary information).

Quantitative Real-Time PCR Analysis: Pellets of 500 *C. elegans* in 0.5 mL 85 mM NaCl were collected (days 2, 5 and 12 of adulthood), mixed with 1 mL TRIzol reagent (Ambion® by Life Technologies, Thermo Fisher Scientific, Waltham, USA) shock frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction. As published elsewhere,[23] the content of isolated RNA was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific) and cDNA synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific), according to the manufacturer's protocol. TaqMan Gene Expression PCR analysis was conducted with half of the recommended volumes of 1:2 diluted cDNA, 20X TaqMan Gene Expression Assays and 2X TaqMan Gene Expression Master Mix (both Applied Biosystems) and DEPC water (Carl Roth GmbH + Co. KG). The respective probes (Applied Biosystems) are listed in supplementary Table S3. Fold-change of gene expression was calculated by applying the $2^{-\Delta\Delta Ct}$ method, normalized to mRNA levels of the house-keeping gene *afd-1* (orthologous to human β actin)[24] and presented in relation to N2 wildtype day 2 control values.

Dopaminergic Neurodegeneration: BY200 and MAB8 were chronically supplemented and propagated until day 2, day 5 and day 12 of adulthood as described above. About 25 worms per condition were anesthetized by picking them into 5 mM levamisole (Sigma-Aldrich Chemie GmbH) on 4% agarose pads on microscope slides. Z-series images of the head neurons were captured with a Leica DMB6 fluorescence microscope (Leica Camera AG, Wetzlar, Germany) equipped with a CTR5 LED lamp and 64x magnification objective applying identical settings and exposure times (40X magnification, 25 ms exposure time, gain 1). Images were processed and edited with Leica LAS X software with Thunder Imager to obtain blur-free detailed overlays of 20 Z-stacks each. Neurodegenerative events per 15 worms were scored according to morphologic characteristics defined as mild (discontinuous signal of dendrites (gaps or breaks, very small blebs) and/or dendritic blebbing), moderate (splinted and/or shrunken soma) or severe (lost soma and/or dendrites).

Statistical Analysis: Statistical analyses and figures were compiled using GraphPad Prism 7 (GraphPad Software, La Jolla, USA). Significances of intraday (# compared to 5 mM Mn, § compared to 0.5 mM Zn and ◊ compared to 1 mM Zn of the respective same age), interday (♦ compared to day 2 and day 5 of the respective same condition) and respective same-day control (*) comparisons were analyzed using one-way ANOVA with Tukey's multiple comparison test. Significant differences are denoted as */#/§/◊: $p < 0.05$, **/##/§§/◊◊: $p < 0.01$ and ***/###/§§§/◊◊◊/♦♦♦: $p < 0.005$. An unpaired Student t-test was applied for strain comparisons and depicted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.005$ compared to respective same condition of N2 wildtype. Unless otherwise stated, experiments were conducted in three biological replicates.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank the DFG Research Unit TraceAge (FOR 2558, BO4103/4-1, HA4318/4-1) for supporting this work. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). M.A. was supported in part by grants from the National Institute of Environmental Health Sciences (NIEHS) R01ES07331 and R01ES10563.

Conflict of Interest

The authors declare no conflict of interests.

Author Contributions

Je.B. designed and performed experiments, conducted data analysis and interpretation. Experimental work was supported by V.M. in developing the feeding scenario and picture editing and M.S. in ICP-MS/MS analysis. S.R.S. and M.A. provided worm strains. Je.B., S.R.S., and Ju.B. wrote the manuscript. S.R.S., M.A., H.H., and T.S. contributed to data interpretation and revised the manuscript critically for important intellectual content. All authors were involved in compiling the manuscript and approved the final version. Ju.B. and T.S. rendered this work possible.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

aging, *C. elegans*, homeostasis, manganese, zinc

Received: December 11, 2020
Revised: February 15, 2021
Published online: March 9, 2021

[1] *United Nations, Department of Economic and Social Affairs, Population Division, World Population Ageing* 2019. (ST/ESA/SER.A/444) **2020**.
[2] A. Y. Chang, V. F. Skirbekk, S. Tyrovolas, N. J. Kassebaum, J. L. Dieleman, *The Lancet Public Health*, https://doi.org/10.1016/S2468-2667(19)30019-2.
[3] a) H. Haase, L. Rink, *Immunity & Ageing: I & A* https://doi.org/10.1186/1742-4933-6-9; b) N. L. Parmalee, M. Aschner, *Neurotoxicology* https://doi.org/10.1016/j.neuro.2016.06.006.
[4] P. Chen, J. Bornhorst, M. Aschner, *Front. Biosci.* https://doi.org/10.2741/4665.
[5] W. Maret, *Int. J. Mol. Sci.* https://doi.org/10.3390/ijms18112285.
[6] European Food Safety Authority (EFSA), *EFS2*, https://doi.org/10.2903/j.efsa.2013.3419.
[7] J. H. Freeland-Graves, T. Y. Mousa, S. Kim, *J. Trace. Elem. Med. Biol.* https://doi.org/10.1016/j.jtemb.2016.05.004.
[8] a) C. Livingstone, *Nutr. Clin. Pract.* https://doi.org/10.1177/0884533617702837; b) D. Santos, C. Batoreu, L. Mateus, A. P. Marreilha Dos Santos, M. Aschner, *Neurotoxicology* https://doi.org/10.1016/j.neuro.2013.10.003.
[9] A. C. Martins, B. N. Krum, L. Queirós, A. A. Tinkov, A. V. Skalny, A. B. Bowman, M. Aschner, *J. Agric. Food Chem.* https://doi.org/10.1021/acs.jafc.0c00641.
[10] Z. Vural, A. Avery, D. I. Kalogiros, L. J. Coneyworth, S. J. M. Welham, *Nutrients* https://doi.org/10.3390/nu12041072.
[11] P. Uciechowski, L. Rink, in *Molecular Basis of Nutrition and Aging* (Eds: M. Malavolta, E. Mocchegiani), Academic Press, **2016**, 551–566.
[12] K. J. Barnham, A. I. Bush, *Curr. Opin. Chem. Biol.* https://doi.org/10.1016/j.cbpa.2008.02.019.
[13] P. Chen, M. R. Miah, M. Aschner, *F1000Res.* https://doi.org/10.12688/f1000research.7431.1.
[14] D. J. Spurgeon, O. A. H. Jones, J.-L. C. M. Dorne, C. Svendsen, S. Swain, S. R. Stürzenbaum, *Sci. Total Environ.* https://doi.org/10.1016/J.SCITOTENV.2010.02.038.

[15] G. Bjørklund, J. Aaseth, A. V. Skalny, J. Suliburska, M. G. Skalnaya, A. A. Nikonorov, A. A. Tinkov, *J. Trace. Elem. Med. Biol.* https://doi.org/10.1016/j.jtemb.2017.02.005.
[16] Q. Ye, J. E. Park, K. Gugnani, S. Betharia, A. Pino-Figueroa, J. Kim, *Metallomics* https://doi.org/10.1039/c7mt00079k.
[17] M. Knez, R. D. Graham, R. M. Welch, J. C. R. Stangoulis, *Crit. Rev. Food Sci. Nutr.* https://doi.org/10.1080/10408398.2015.1050483.
[18] S. J. Garcia, K. Gellein, T. Syversen, M. Aschner, *Toxicol. Sci.* https://doi.org/10.1093/toxsci/kfl017.
[19] J.-S. Park, N. F. Blair, C. M. Sue, *Mov. Disord.* https://doi.org/10.1002/mds.26243.
[20] a) A. Olsen, M. S. Gill, in, *Healthy Ageing and Longevity* (Eds: A. Olsen, M. S. Gill), Springer International Publishing, Cham **2017**, p. 1–7; b) L. A. Herndon, C. Wolkow, D. H. Hall, WormAtlas https://doi.org/10.3908/wormatlas.8.4; c) F. A. Soares, D. A. Fagundez, D. S. Avila, in *Advances in Neurobiology*, (Eds: M. Aschner, L. G. Costa), Springer International Publishing, Cham, **2017**, p. 355–383; d) J. J. Collins, C. Huang, S. Hughes, K. Kornfeld, *WormBook*, https://doi.org/10.1895/wormbook.1.137.1.
[21] S. Brenner, *Genetics* **1974**, *77*, 71.
[22] J. Baesler, J. F. Kopp, G. Pohl, M. Aschner, H. Haase, T. Schwerdtle, J. Bornhorst, *J. Trace Elem. Med. Biol.* https://doi.org/10.1016/j.jtemb.2019.05.005.
[23] C. Neumann, J. Baesler, G. Steffen, M. M. Nicolai, T. Zubel, M. Aschner, A. Bürkle, A. Mangerich, T. Schwerdtle, J. Bornhorst, *J. Trace Elem. Med. Biol.* https://doi.org/10.1016/j.jtemb.2019.09.001.
[24] K. J. Livak, T. D. Schmittgen, *Methods* https://doi.org/10.1006/meth.2001.1262.
[25] C. Au, A. Benedetto, J. Anderson, A. Labrousse, K. Erikson, J. J. Ewbank, M. Aschner, *PLoS One* https://doi.org/10.1371/journal.pone.0007792.
[26] S. Chakraborty, P. Chen, J. Bornhorst, T. Schwerdtle, F. Schumacher, B. Kleuser, A. B. Bowman, M. Aschner, *Metallomics* https://doi.org/10.1039/c5mt00052a.
[27] D. E. Davis, H. C. Roh, K. Deshmukh, J. J. Bruinsma, D. L. Schneider, J. Guthrie, J. D. Robertson, K. Kornfeld, *Genetics* https://doi.org/10.1534/genetics.109.103614.
[28] H. C. Roh, S. Collier, K. Deshmukh, J. Guthrie, J. D. Robertson, K. Kornfeld, *PLoS Genet.* https://doi.org/10.1371/journal.pgen.1003522.
[29] R. Bofill, R. Orihuela, M. Romagosa, J. Domènech, S. Atrian, M. Capdevila, *FEBS J.* https://doi.org/10.1111/j.1742-4658.2009.07417.x.
[30] S. Zeitoun-Ghandour, J. M. Charnock, M. E. Hodson, O. I. Leszczyszyn, C. A. Blindauer, S. R. Stürzenbaum, *FEBS J.* https://doi.org/10.1111/j.1742-4658.2010.07667.x.
[31] a) J. Bornhorst, *T. Schwerdtle in Issues in Toxicology* (Eds: L. Costa, M. Aschner), Royal Society of Chemistry, Cambridge **2014**, p. 604; b) S. R. Lee, *Oxid. Med. Cell. Longev.* https://doi.org/10.1155/2018/9156285; c) K. M. Erikson, M. Aschner, *Met. Ions Life Sci.* https://doi.org/10.1515/9783110527872-016.
[32] C. López-Otín, M. A. Blasco, L. Partridge, M. Serrano, G. Kroemer, *Cell* https://doi.org/10.1016/j.cell.2013.05.039.
[33] S. W. A. An, M. Artan, S. Park, O. Altintas, S.-J. V. Lee, in *Healthy Ageing and Longevity* (Eds: A. Olsen, M. S. Gill), Springer International Publishing, Cham **2017**, p. 63.
[34] E. B. Kayser, P. G. Morgan, C. L. Hoppel, M. M. Sedensky, *J. Biol. Chem.* https://doi.org/10.1074/jbc.M011066200.
[35] A. Benedetto, C. Au, D. S. Avila, D. Milatovic, M. Aschner, *PLoS Genet.* https://doi.org/10.1371/journal.pgen.1001084.
[36] W. Springer, T. Hoppe, E. Schmidt, R. Baumeister, *Hum. Mol. Genet.* https://doi.org/10.1093/hmg/ddi371.
[37] I. M. Klang, B. Schilling, D. J. Sorensen, A. K. Sahu, P. Kapahi, J. K. Andersen, P. Swoboda, D. W. Killilea, B. W. Gibson, G. J. Lithgow, *Aging (Albany NY)* https://doi.org/10.18632/aging.100689.
[38] L. Sauzéat, A. Laurençon, V. Balter, *Metallomics* https://doi.org/10.1039/c7mt00318h.
[39] J. Kumar, T. Barhydt, A. Awasthi, G. J. Lithgow, D. W. Killilea, P. Kapahi, *PLoS One* https://doi.org/10.1371/journal.pone.0153513.
[40] J. Baudry, J. F. Kopp, H. Boeing, A. P. Kipp, T. Schwerdtle, M. B. Schulze, *Eur. J. Nutr.* https://doi.org/10.1007/s00394-019-02143-w.
[41] S. Angeli, T. Barhydt, R. Jacobs, D. W. Killilea, G. J. Lithgow, J. K. Andersen, *Metallomics* https://doi.org/10.1039/c4mt00168k.
[42] F. C. Wedler, B. W. Ley, *Neurochem. Res.* https://doi.org/10.1007/BF01208583.
[43] C. J. Mercadante, C. Herrera, M. A. Pettiglio, M. L. Foster, L. C. Johnson, D. C. Dorman, T. B. Bartnikas, *BioMetals* https://doi.org/10.1007/s10534-016-9924-6.
[44] M.-T. Chen, G.-W. Cheng, C.-C. Lin, B.-H. Chen, Y.-L. Huang, *Biol. Trace Elem. Res.* https://doi.org/10.1385/BTER:110:2:163.
[45] M.-B. Troadec, D. M. Ward, E. Lo, J. Kaplan, I. de Domenico, *Blood* https://doi.org/10.1182/blood-2010-04-278614.
[46] C. J. Mitchell, A. Shawki, T. Ganz, E. Nemeth, B. Mackenzie, *Am. J. Physiol. Cell Physiol.* https://doi.org/10.1152/ajpcell.00348.2013.
[47] H. C. Roh, S. Collier, J. Guthrie, J. D. Robertson, K. Kornfeld, *Cell Metab.* https://doi.org/10.1016/j.cmet.2011.12.003.
[48] K. Warnhoff, H. C. Roh, Z. Kocsisova, C.-H. Tan, A. Morrison, D. Croswell, D. L. Schneider, K. Kornfeld, *PLoS Biol.* https://doi.org/10.1371/journal.pbio.2000094.
[49] S.-T. Li, H.-Q. Zhao, P. Zhang, C.-Y. Liang, Y.-P. Zhang, A.-L. Hsu, M.-Q. Dong, *Aging Cell* https://doi.org/10.1111/acel.12896.
[50] K. Kobayashi, J. Kuroda, N. Shibata, T. Hasegawa, Y. Seko, M. Satoh, C. Tohyama, H. Takano, N. Imura, K. Sakabe, H. Fujishiro, S. Himeno, *J. Pharmacol. Exp. Ther.* https://doi.org/10.1124/jpet.106.112912.
[51] J. Xiao, Q. Rui, Y. Guo, X. Chang, D. Wang, *J. Environ. Sci.* https://doi.org/10.1016/S1001-0742(08)62350-5.
[52] M. R. C. Schetinger, T. V. Peres, L. P. Arantes, F. Carvalho, V. Dressler, G. Heidrich, A. B. Bowman, M. Aschner, *Toxicology* https://doi.org/10.1016/j.tox.2018.10.006.
[53] L. Mezzaroba, D. F. Alfieri, A. N. Colado Simão, E. M. Vissoci Reiche, *Neurotoxicology* https://doi.org/10.1016/j.neuro.2019.07.007.
[54] E. V. Stelmashook, N. K. Isaev, E. E. Genrikhs, G. A. Amelkina, L. G. Khaspekov, V. G. Skrebitsky, S. N. Illarioshkin, *Biochemistry (Mosc)* https://doi.org/10.1134/S0006297914050022.
[55] P. A. Lewis, J. E. Spillane, in *The Molecular and Clinical Pathology of Neurodegenerative Disease*, Elsevier, **2019**, p. 83–121.
[56] D. Truban, X. Hou, T. R. Caulfield, F. C. Fiesel, W. Springer, *J. Parkinsons. Dis.* https://doi.org/10.3233/JPD-160989.
[57] J. F. Cooper, E. Machiela, D. J. Dues, K. K. Spielbauer, M. M. Senchuk, J. M. van Raamsdonk, *Sci. Rep.* https://doi.org/10.1038/s41598-017-16637-2.
[58] D. Pretsch, J. M. Rollinger, A. Schmid, M. Genov, T. Wöhrer, L. Krenn, M. Moloney, A. Kasture, T. Hummel, A. Pretsch, *Sci. Rep.* https://doi.org/10.1038/s41598-020-68561-7.