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Deregulation of biometal homeostasis: the missing link for neuronal ceroid lipofuscinoses?

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ABSTRACT
Neuronal ceroid lipofuscinoses (NCLs), a group of genetically distinct fatal neurodegenerative disorders with no treatment or cure, are clinically characterised by progressive motor and visual decline leading to premature death. While the underlying pathological mechanisms are yet to be precisely determined, the diseases share several common features including inflammation, lysosomal lipofuscin deposits and lipid abnormalities. An important hallmark of most common neurodegenerative disorders including Alzheimer's, Parkinson's and motor neuron diseases is deregulation of biologically active metal homeostasis. Metals such as zinc, copper and iron are critical enzyme cofactors and are important for synaptic transmission in the brain, but can mediate oxidative neurotoxicity when homeostatic regulatory mechanisms fail. We previously demonstrated biometal accumulation and altered biometal transporter expression in 3 animal models of CLN6 NCL disease. In this study we investigated the hypothesis that altered biometal homeostasis may be a feature of NCLs in general using 3 additional animal models of CLN1, CLN3 and CLN5 disease. We demonstrated significant accumulation of the biometals zinc, copper, manganese, iron and cobalt in these mice. Patterns of biometal accumulation in each model preceded significant neurodegeneration, and paralleled the relative severity of disease previously described for each model. Additionally, we observed deregulation of transcripts encoding the anti-oxidant protein, metallothionein (Mt), indicative of disruptions to biometal homeostasis. These results demonstrate that altered biometal homeostasis is a key feature of at least 4 genetically distinct forms of NCL disease.

INTRODUCTION

NCLs are a group of inherited fatal lysosomal storage disorders (LSDs) with neurological involvement. There are currently 14 recognized forms of NCLs that are distinguished by genetic deficit (CLN1-14) and different ages of onset ranging from congenital to adult, although most variants manifest during childhood. Symptoms also vary in severity and rate of progression, but commonly include epileptic seizures, progressive dementia, blindness and motor decline leading to paralysis and premature death (reviewed in [1]).

The development of mouse models that closely recapitulate relevant disease features has been instrumental to investigation of disease mechanisms, and assessment of treatment efficacy. Infantile NCL (INCL) is caused by mutations in the gene encoding palmitoyl protein thioesterase 1, PPT1/CLN1, a lysosomal enzyme involved in depalmitoylation of S-acylated proteins.[2] The severe nature and early onset of CLN1 disease is replicated in *Cln1* knockout (ko) mice lacking exon 4,[3] that show early loss of vision by 3.5 months, clasping and seizures by 4 months and myoclonic jerks by 6 months of age. The progression of the disease results in a hind limb paralysis at the average age of 5.2 months, followed by death at approximately 6.5 months. The brain mass of *Cln1* ko mice is remarkably reduced due to cortical atrophy. Storage material, a prominent pathological feature in NCL patients,[1] is abundant in the brain at the age of 3 months, and continues to accumulate in the brain and retina until the age of 6 months. Another *Cln1* ko mouse model containing a neomycin resistance gene inserted instead of exon 9, lacks an obvious progressive visual impairment phenotype, although myoclonic jerks and clasping behaviors are similar to those observed in the exon 4 ko animals.[4]

By contrast, the most common NCL variant, called Juvenile NCL (JNCL) or Batten disease is a milder form of disease. JNCL is caused by defects in *CLN3*, which encodes a putative transporter localized to multiple cellular compartments, postulated to be involved in regulation of intracellular pH and osmotic shock responses.[5, 6] A number of knock in and ko mice modeling CLN3 disease have been generated. The most widely used to date are *Cln3ΔEx7-8* and *Cln3ΔEx1-6* ko mice.[7, 8] Similar to disease progression in patients, these murine models exhibit milder symptoms and later onset compared to the *Cln1* mice. Storage material is evident prior to birth in *Cln3ΔEx7-8* mice, expressing the most common human *CLN3* mutation, a ~1kb deletion.[7] The mice display gait abnormalities, clasping behavior, reduced retinal photoreceptor numbers and an 80% survival rate by 12 months of age.[7] *Cln3ΔEx1-6* mice are largely asymptomatic until 12 months, although autofluorescent cortical inclusions and decreased cortical volume due to loss of parvalbumin-positive interneurons are evident from 5 and 7 months of age, respectively.[8] Moreover, a later study reported that *Cln3ΔEx1-6* mice displayed impaired motor function from post-natal day 14, and significant inflammatory phenotypes including activated cerebellar Bergmann glia.[9]

Variant late infantile NCL (vLINCL) is caused by mutations in *CLN5,* encoding a lysosomal glycoprotein[10] of unknown function with a putative role in ceramide synthesis.[11] vLINCL is milder than CLN1, but more aggressive than CLN3 disease. Similarly, the mouse model for CLN5 disease, which encodes a truncated protein due

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1   to the deletion of exon 3,[12] displays an intermediate phenotype compared to *Cln1* and
2   *Cln3* ko animals. *Cln5* ko mice lose vision at the age of 5.2 months, but show no
3   motor dysfunction or brain atrophy.[12] The presence of storage material, with a
4   characteristic vLINCL fingerprint and curvilinear profile begins at the age of 3
5   months, and accumulation of autofluorescence throughout the brain is evident by 6
6   months.
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8   These mouse models, as well as others, with genetic defects in CLN2/TPP1, CLN6,
9   CLN8, CLN10/Cathepsin D (reviewed in [13]), have yielded an immense amount of
10 biochemical and mechanistic information on the similarities and differences between
11 different forms of NCLs. It is now established that the NCL gene products are
12 localized throughout the cell: in the lysosome (CLN1/PPT1, CLN2/TPP1, CLN5,
13 CLN10/Cathepsin D, CLN13/Cathepsin F CLN12/ATP13a2, CLN7), the endoplasmic
14 reticulum (CLN6, CLN8), the secretory pathway (CLN3, CLN4), the cytoplasm
15 (CLN14/KCTD7) or are extracellular (CLN11/progranulin; reviewed in [14]). In
16 contrast to most other LSDs, only 4 of the NCL-causative genes encode lysosomal
17 enzymes (CLN1/PPT1, CLN2/TPP1, CLN10/Cathepsin D, CLN13/Cathepsin F). The
18 functions of the majority of the NCL proteins remain unknown, although
19 CLN12/ATP13a2 and CLN14/KCTD6 are postulated to act as ion channels. Unlike
20 most classical LSDs, lysosomal storage of lipofuscin material in NCLs is not directly
21 associated with defective lysosomal enzymes that prevent degradation of particular
22 substrates, and is also not predictive of regional neuronal pathology.[15] Another
23 common pathological feature for this group of genetically distinct but clinically
24 related disorders is inflammation;[3, 12] activated brain astrocytes and glia are evident
25 prior to birth in some NCL models and spatially parallel subsequent neuronal
26 pathology.[15] Impaired cellular lipid metabolism has also been implicated in several
27 NCL forms; reports in both patients and mouse NCL models indicate the presence of
28 altered serum lipid profiles,[16] elevated cholesterol[17, 18] and aberrant storage of
29 phospholipids.[19] Moreover, CLN1/PPT1 and CLN3 have been detected in lipid
30 rafts,[20, 21] consistent with a role in maintenance of cellular lipid dynamics. Interactions
31 between several subsets of NCL proteins, including those between CLN5 and
32 CLN1/PPT1, CLN2/TPP1, CLN3, CLN6 or CLN8,[22] are indicative of common
33 pathogenic mechanisms, but these are yet to be precisely delineated. This is further
34 confounded by the cell-specific physiological central nervous system (CNS)
35 expression levels of each NCL protein – CLN1 is primarily expressed in neurons and
36 astrocytes, while CLN3 levels are greatest in neurons and microglia,[23] and microglia
37 express a high amount of CLN5.[18] Hence, while it appears that convergent molecular
38 processes may account for some pathological NCL traits, the current understanding of
39 the mechanisms involved is limited.
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41 A common hallmark of neurodegeneration is the deregulation of biologically active
42 metal homeostasis.[24] Tight cellular control over metals such as zinc, copper and iron
43 is critical – these metals comprise enzyme cofactors of over 10% of all proteins, [25] but
44 can induce toxic oxidative damage if concentrations of labile metal pools rise.[26] The
45 particular vulnerability of the brain to metal dyshomeostasis is supported by the vast
46 number of neurodegenerative diseases with metal handling abnormalities, including
47 Alzheimer's, Parkinson's and motor neuron diseases, and the demonstrated
48 therapeutic efficacy of metal-targeted treatments.[27-30] We have previously shown that
49 changes to biometal homeostasis also drive pathology in 3 natural models of CLN6
50 NCL,[31, 32] involving aberrant expression of the ER/Golgi resident biometal

transporter, Zip7 (manuscript submitted). Moreover, we reported that biometal accumulation in CLN6 tissues involved dramatic compensatory upregulation of the metal sequestering protein, Mt.[32] Here, using an additional 3 genetically distinct NCL mouse models, we demonstrate that the aberrant metal accumulation and Mt overexpression phenotype is common to multiple forms of NCLs, including CLN1, CLN3 and CLN5 variants. Aside from inflammation, lipid metabolism and lipofuscin accumulation, alterations to metal homeostasis appear to represent an additional phenotypic characteristic common to multiple forms of NCLs, including variants caused by both soluble and membrane bound gene products that are present in different cellular compartments.

RESULTS

Biometals accumulate in the brains of NCL model mice

We previously showed that aberrant biometal accumulation contributes to disease pathology in 3 natural animal models of CLN6 disease. To determine whether impaired biometal homeostasis is a pathological feature common to multiple forms of NCLs, we investigated the metal content in CNS tissues of mice modeling CLN1, CLN3 and CLN5 diseases. At least one presymptomatic and one post-symptom onset age was chosen to perform analyses for each model. *Cln1* ko mice are phenotypically normal until 3.5 months of age, when visual dysfunction first becomes apparent (Table 1). The lifespan of these mice is approximately 6.5 months. *Cln5* ko mice develop vision loss after 5 months of age, but do not exhibit motor deficits. *Cln3* ko mice display symptoms after 12 months, but some neuronal loss is evident by 7 months. We therefore chose the following ages for analysis: for *Cln1* ko mice- 3,4,and 5 months; for *Cln5* ko mice – 3,4,5 and 7 months; and for *Cln3* ko mice- 6 and 12 months. We examined the following CNS tissue regions in *Cln1* and *Cln5* ko mice: olfactory bulb, cortex, cerebellum, hippocampus and spinal cord. In *Cln3* ko mice, we analysed the brainstem, cortex, thalamus, and spinal cord. The thalamus was included for *Cln3* ko mice, as this was previously reported to be an important region for pathology in this model at 12 months of age.[5] Due to limited material, not all analyses could be performed on all animals and all ages. The levels of zinc, copper, manganese, cobalt and iron were significantly elevated in NCL model mice. Due to the complexity of the current study, we focused only on the changes observed to metals that are primarily protein-bound in biological tissues. Therefore, the examination of changes to additional, biologically important free metals, including calcium, magnesium, potassium and sodium is outside the scope of this study and will be reported elsewhere.

A significant increase in zinc concentrations was detected in the olfactory bulb in 3 month-old *Cln1* ko mice (Fig. 1A), progressing to the cortex and cerebellum by 4 months of age (Fig. 1B). By 5 months of age, significantly elevated zinc was detected in all brain regions analysed in these mice, the most dramatic in the olfactory bulb with a 90% increase above the levels in wild-type mice (Fig. 1C). As these mice succumb to disease at ~6.5 months, no further tissue sampling was performed after 5 months. *Cln5* ko mice, which develop a milder form of disease, displayed a trend of increased metal content in all CNS regions by 5 months of age, which reached statistical significance by 7 months of age (Fig. 1D). Zinc concentrations were increased by between 34% in the hippocampus to 130% in the olfactory bulb. No significant changes to zinc content were detected in CLN3 mice, although a trend

towards increased zinc in the cortex and thalamus of 6 month-old mice was observed (Fig. 1E-F).

Changes in CNS copper concentrations in *Cln1* and *Cln5* ko mice largely paralleled the changes observed for zinc. In *Cln1* ko mice, copper was increased significantly in the olfactory bulb at 3 months (Fig. 2A), in the cortex and cerebellum at 4 months (Fig. 2B) and within all brain structures tested at 5 months, except the cerebellum, where the observed increase did not reach statistical significance (Fig. 2C). The rise in copper concentrations was 114% greater than in controls in the olfactory bulb. A trend of elevation in copper concentrations was observed in *Cln5* ko mice from 4 months of age, with statistically significant rises in copper being evident in the hippocampus in 5 month-old mice (Fig 2B-C). By 7 months of age, CNS-wide dramatic increases of 80-220% in copper concentrations were observed in CLN5 mice (Fig. 2D). In 6 month-old *Cln3* ko mice, copper levels were significantly increased in the thalamus, and an overall increase was observed in CNS tissues (Fig. 2E). By 12 months of age, however, copper levels were not significantly different to those of wild-type mice (Fig. 2F).

Similarly, manganese concentrations were progressively elevated in *Cln1* and *Cln5* ko mice over the course of disease. Analogous to copper and zinc, elevated manganese content was first detected in the olfactory bulb of 3 month-old *Cln1* ko mice (Fig. 3A), then the cortex, cerebellum and spinal cord at 4 months (Fig. 3B) and all regions by 5 months, except the hippocampus where the rise did not reach significance (Fig. 3C). Manganese accumulation was detected in the cortex, olfactory bulb and spinal cord in *Cln5* ko mice at 7 months of age (Fig. 3D). However, no changes to manganese concentrations were observed in *Cln3* ko animals (Fig. 3E-F).

The early alterations to iron levels in *Cln1* ko mice followed the regional pattern described for zinc, copper and manganese, however, by 5 months of age, iron was only increased in the cerebellum and olfactory bulb (Fig. 4A-C). Similarly, the most pronounced iron elevation in *Cln5* ko mice was observed in the olfactory bulb, cerebellum and spinal cord at 7 months of age (Fig. 4D). While iron concentrations did not differ between WT and *Cln3* ko animals at 6 months (Fig. 4E), at 12 months, there was a significant overall increase in iron content in the CNS of *Cln3* ko mice (Fig. 4E-F).

Striking changes to cobalt concentrations were evident in *Cln1*, *Cln3* and *Cln5* ko mice. Similar to the metals described above, cobalt accumulation began in the olfactory bulb in 3 month-old *Cln1* ko animals, progressing to the cortex, cerebellum and spinal cord by 4 months, and all CNS tissues except for the hippocampus by 5 months (Fig. 5A-C). Interestingly, cobalt levels were substantially reduced in hippocampi of 3 month-old *Cln1* ko mice, the only metal we found to be deficient in any tissue tested. Widespread rises in cobalt concentrations in the cerebellum, cortex and olfactory bulb at 5 months were the earliest metal changes detected in *Cln5* ko mice, and were progressively increased in every tissue except the hippocampus by 7 months (Fig. 5C-D). Cobalt concentrations were also increased in the CNS of 6 month-old *Cln3* ko mice, but stabilized by 12 months of age (Fig. 5E-F). Cobalt content in the thalamus was below the level of detection in either WT or *Cln3* ko animals.

Our previous reports indicated that the heart and liver were also sites of biometal accumulation in 3 natural CLN6 sheep and mouse models.[31, 32] However, we detected no differences in the concentrations of zinc, copper, manganese, iron or cobalt in the livers or hearts of *Cln1, Cln3* or *Cln5* ko mice at any age (Fig. S1-2), suggesting that aberrant biometal homeostasis originates specifically in CNS tissues in these models. Taken together, these data demonstrate dramatic and broadly progressive regional CNS accumulation of biometals throughout the disease course in *Cln1* and *Cln5* ko mice, and more subtle regional changes to copper, iron and cobalt in *Cln3* ko mice, highlighting the complex nature of spatio-temporal biometal disturbances in the brains of animals with NCL diseases.

Elevated Mt expression in NCL model mice

We next examined expression of transcripts encoding various isoforms of the metal sequestering and anti-oxidant acute phase protein, Mt, to verify early disease-associated changes to metal homeostasis in NCL model mice. While it would be advantageous to also measure Mt protein levels, most commercially available antibodies to Mt do not distinguish between Mt isoforms and detect bands ranging from 28-35kDa, whereas the molecular weight of Mt ranges from 5 to 14 kDa. Moreover, there is no evidence of post-transcriptional or post-translational regulation of Mt isoforms in the brain. Therefore quantitative changes to *Mt* transcripts provide an additional indicator of disrupted metal homeostasis. We chose to perform analyses for *Cln1* and *Cln5* ko mice in the cortical region, due to the significant metal changes observed in the cortex (Fig. 1-5), previously demonstrated similarities between cortical transcriptional profiles in *Cln1* and *Cln5* ko mice,[33] and, because the cortex is the initial site of significant neuron loss in *Cln5* ko mice.[34] For *Cln3* ko mice, the cerebellum was chosen as it was reported to represent an important region of pathology in these animals[9] and JNCL patients.[35] We also compared *Mt* expression in the *Cln1, Cln3* and *Cln5* ko mice with *Cln6 nclf* mice (Table 1). We previously reported changes to metal homeostasis in the cortex and spinal cord in 3 month-old *Cln6 nclf* mice, whereas the cerebellum was a later site of dramatic metal accumulation.[31] We therefore analysed *Mt* gene expression in cortices, cerebella and spinal cords of *Cln6 nclf* mice.

The *Mt1* and *Mt2* transcripts were significantly upregulated in 5 month-old *Cln1* ko mice, and were also both increased by 1.9 fold in 5 month-old *Cln5* ko mice, although this did not reach statistical significance (Fig. 6A). *Mt3* mRNA was also 2.2 and 1.4 fold elevated, respectively, in *Cln1* and *Cln5* ko mice. These data are consistent with increased cortical copper and zinc, both capable of strongly inducing *Mt* transcription. An overall significant increase of *Mt* expression was observed in the cerebella of 6 month-old CLN3 mice, although this did not reach statistical significance for any single *Mt* transcript (Fig. 6B). We also compared *Mt* expression in the cortex, cerebellum and spinal cord of 3 month-old pre-symptomatic *Cln6 nclf* mice. Interestingly, while no significant changes to *Mt1* or *Mt3* transcription were detected (Fig. 6C-E), we observed significant deregulation of *Mt2* in the brains of *Cln6 nclf* mice (Fig. 6E, F). In the spinal cord, where we previously reported zinc and manganese accumulation at this age,[31] *Mt2* was greater than 2 fold overexpressed, whereas in the cerebellum, the site of inflammatory responses at this age (A. Grubman, unpublished observations), *Mt2* expression was dramatically reduced compared to control mice. Aberrant *Mt* expression in multiple models further emphasizes the deregulation of CNS biometal homeostasis in NCL model mice.

Metal transport protein expression in NCL model mice

Aside from cytoplasmic buffering of excess labile metal pools by Mt,[36] subcellular metal trafficking dynamics are mediated by membrane-bound metal transport proteins.[37] We previously reported that the ER/Golgi-resident zinc influx transporter, Zip7, was aberrantly expressed in two natural CLN6 sheep models (manuscript submitted) and the natural *Cln6 nclf* mouse model.[31] We also detected changes to other transporters of the Zip/SLC39A and ZnT/SLC30A families that are primarily known to transport zinc, but not to the copper transporters CTR1, CTR2 or ATP7A/B, despite the greater changes to copper in the brains of those animals. We therefore investigated whether changes to biometal transport proteins of the Zip (Zip7, 8,14) and ZnT (ZnT5, 6,7) families also occur in *Cln1, Cln3* and *Cln5* ko mice. We detected no changes in expression of any metal transport proteins tested in the cerebella of 5 and 7 month-old *Cln1* or *Cln5* ko mice, respectively (Fig 7). We were unable to detect expression of Zip14, ZnT5 or ZnT7 protein in either 5 month-old WT Rcc or *Cln1* animals. Given that expression is detectable in 7 month-old WT Rcc animals (Fig. 7), it is possible that these proteins are regulated in an age-dependent manner in the brains of the Rcc strain of mice. However, a ~30kDa Zip7-reactive band was specifically upregulated in CLN5 mice. This corresponds to a predicted low molecular weight isoform of Zip7 (Ensembl Havana Protein ID ENSMUSP00000133146) containing a histidine-rich region and a Zip domain.

GAPDH and tubulin expression did not correlate to total protein content in *Cln3* ko mice (data not shown) which may reflect previously reported impaired ATP production in human CLN3 disease[38] and cytoskeletal abnormalities in *Cln3* mouse neurons,[39] precluding from using these markers as loading controls. Therefore, for *Cln3* ko mice, total ERK was used as a loading control. No significant differences were detected in the expression levels of any of the Zip or ZnT proteins analysed. However, there was a trend towards increased expression of ZnT7 in the *Cln3* ko cerebella. Together, the data demonstrate that biometal accumulation in CNS tissues in *Cln1, Cln3,* and *Cln5* ko mice occurs in the absence of significant tissue-level alterations to metal transporter expression.

DISCUSSION

We previously reported accumulation of zinc, copper, iron, manganese and cobalt in the disease-affected occipital lobe in 2 sheep models of CLN6 disease and the mouse *Cln6 nclf* model.[31, 32] Deregulation of metal homeostasis was associated with early and progressive changes to the ER/Golgi localized metal transporter, Zip7, as well as subsequent changes to other transporters including Zip8, Zip14, ZnT6 and ZnT7 after symptom onset (manuscript submitted). In this study, we sought to determine whether deregulation of biometal homeostasis is a common feature to multiple forms of NCLs. We therefore investigated metal metabolism in 3 additional genetic NCL diseases with different molecular disturbances and different underlying pathogenic mechanisms. Indeed, zinc, copper, iron, manganese and cobalt levels were elevated in *Cln1, Cln3* and *Cln5* ko animals, indicating that impaired metal homeostasis is not a CLN6 disease-specific phenotype. Consistent with this, a previous microarray study reported changes in ZnT6 transcripts in *Cln3ΔEx7-8* and *Cln6 nclf* mouse cerebellar neuron precursor cell lines.[40] Moreover, two of the genes mutated in NCLs are metal

1 transporters: ATP13A2, the cause of an adult onset form of NCL, Kuf's disease,[41-43] is
2 a lysosomal cation transporter, and CLN14 is postulated to encode a potassium
3 channel,[44] demonstrating metal transport abnormalities in two additional NCL
4 variants. Together, these studies and our data demonstrate common molecular defects
5 potentially indicating convergent pathogenic processes in these related but distinct
6 disorders.
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8 Zinc is critical for synaptic transmission and is bound by over 3000 proteins, while
9 labile zinc levels are kept low by transport and buffering systems to prevent
10 neurotoxicity. Unregulated labile zinc overload in particular organelles can cause cell
11 death via oxidative induction of mitochondrial membrane depolarization or
12 autophagy-mediated lysosomal dysfunction.[45, 46] However, localized zinc deficiencies
13 are also damaging to neuronal function, as ZnT3 ko mice lacking synaptic zinc
14 display cognitive disabilities,[47] Thus there is a "set point" or optimal labile zinc
15 concentration in each organelle, which can vary from sub-picomolar in the ER and
16 Golgi,[48] low picomolar in the cytoplasm,[49] to high micromolar in synaptic vesicles.[50]
17 Specifically, the ER or Golgi may be highly vulnerable to uncontrolled zinc rises, as
18 we previously reported in *Cln6 nclf* mice.[31] Moreover, trafficking of multiple metals
19 may be linked at the molecular level. For instance, several zinc transporters were
20 reported to be promiscuous to manganese,[51] and are known to bind copper and/or
21 cobalt, although may not actively transport the latter two metals. It is therefore not
22 surprising that the distributions of these metals throughout NCL model CNS tissue are
23 largely similar. Moreover, studies have also shown a close relationship between the
24 brain homeostasis of copper, zinc and iron.[52]
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26 Elevated concentrations of brain metals as observed in *Cln1, Cln3* and *Cln5* brains are
27 likely to have pleiotropic consequences in NCL cells. Lipofuscin deposition is
28 enhanced in the presence of iron and copper,[53] and lysosomal copper is reported to
29 trigger ROS formation, lysosomal membrane permeabilisation and cell death.[54] Iron
30 has been reported to induce pro-inflammatory responses in microglia,[55] and
31 inflammation in turn potentiates cellular iron retention,[56] thereby creating a feedback
32 loop of metal-deregulation. Moreover, brain iron elevation either in mice fed a high-
33 iron diet, or *Hfe* ko mice with a genetic predisposition to brain iron accumulation
34 results in altered transcription of 5 different NCL-causative genes.[57, 58] Manganese
35 can enhance inflammatory cytokine production,[59] and is neurotoxic – overexposure
36 has been linked to parkinsonism (reviewed in [60]). The dynamic deregulation of cobalt
37 observed here may suggest that directional cobalt transport occurs between different
38 brain regions, or that olfactory neurons, a reported entry route for environmental
39 cobalt,[61] are more susceptible to cobalt overload. Cobalt overload has been reported
40 to induce ROS production in astrocytes and mitochondrial dysfunction in neuronal
41 PC12 cells,[62, 63] common features of NCL diseases. However, deficiency of cobalamin
42 (Vitamin B12), requiring cobalt as a cofactor, has been linked to inflammation and
43 myelin damage (reviewed in [64]). It is possible that deficiency of cobalt as observed in
44 the hippocampus of 3 month-old *Cln1* ko mice (Fig. 5A) may contribute to the myelin
45 damage reported in these animals.[65] Therefore impaired biometal homeostasis is
46 capable of initiating and/or exacerbating deregulation of multiple cellular processes
47 that are key pathological features in NCLs, including lipofuscin accumulation,
48 oxidative stress, inflammation, demyelination and deregulation of NCL-causative
49 genes.
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1 Copper and zinc both induce robust Mt production through activation of the metal
2 responsive transcription factor MTF1,[66] thus Mt transcript elevation can be used as a
3 surrogate marker of metal accumulation. In the present study, we observed significant
4 induction of *Mt1* and *Mt2* in *Cln1* ko mouse cortices, as well as an overall trend in
5 upregulation of all 3 *Mt* isoforms in *Cln1, Cln3* and *Cln5* ko mice. This indicates a
6 probable attempt to counteract metal-induced stress via induction of the metal-
7 sequestering Mts. However, elevated metals may be restricted to specific subcellular
8 pools, as previously described for *Cln6 nclf* mice.[31] Therefore, increased metal
9 content may be sensed as metals transit through the cytoplasm, inducing Mt. The
10 altered metal transporter protein expression in some cells may promote metal
11 accumulation in specific organelles such as the ER or lysosomes, where cytoplasmic
12 Mt may not access aberrantly compartmentalized excess metal pools. Thus Mt may
13 not adequately prevent metal-induced toxicity via sequestration of excess
14 accumulated metals. Mt induction may also function as a double-edged sword. While
15 compensatory upregulation results in a higher metal-buffering capacity, this increased
16 capacity for cellular metal retention may increase oxidant-releasable intracellular
17 metal stores.[67] This in turn may be detrimental to cells; zinc liberated from Mt3 upon
18 oxidative stress has been linked to neurotoxicity.[68, 69] Interestingly, we observed a
19 dramatic decrease of *Mt2* transcripts in *Cln6 nclf* cerebella (Fig. 6D). To date only a
20 single transcriptional repressor of *Mt2* has been reported,[70] whereas Mts are activated
21 by various signals including inflammation, increased metal content, oxidative stress
22 and infection and mediate anti-oxidant, neuroregenerative, anti-inflammatory and
23 neuroprotective actions in the brain (reviewed in [66]). As demonstrated here for *Cln6*
24 *nclf* brains, spatiotemporal *Mt* expression is a dynamic process- significantly
25 increased *Mt* expression is indicative of stress responses, whereas reduced *Mt*
26 expression is likely to exacerbate neurodegeneration, inflammation and metal
27 dyshomeostasis.

29 In this study, we examined the levels of the Zip and ZnT transporters, as these were
30 significantly altered in CLN6 disease. No differences were observed in Zip or ZnT
31 expression except for a predicted low molecular mass isoform of Zip7 in CLN5 mice.
32 However, as the underlying causes of biometal accumulation may be different for
33 each NCL variant, cellular expression of copper and iron transporters will be
34 investigated in future studies. Moreover, as metal transporters are differentially
35 expressed in various CNS cell populations,[31] and neurodegeneration in NCLs is
36 highly regional, changes to metal transporter expression may not be adequately
37 detected by bulk analyses. Our previous work indicates that examination of metal
38 changes in particular organelles may provide a more accurate picture of subcellular
39 metal dyshomeostasis, as altered metal and metal transporter content were detected by
40 cellular fractionation and ICP-MS analysis where they were not detectable by bulk
41 measurements alone in *Cln6 nclf* mice.[31] We plan to investigate the metal handling
42 dynamics in NCL cells and tissues from other CLN models using a combination of
43 techniques capable of measuring metals at subcellular resolution, including
44 synchrotron X-ray fluorescence microscopy and fluorescent metal sensors that can be
45 targeted to specific organelles.[48, 71, 72]

47 Both cortical and cerebellar abnormalities have been reported in NCL patients and
48 animal models,[9, 34] and the thalamus is an important pathological region in CLN3
49 disease [35]. A recent study noted the complex progression of atrophy in the brains of
50 the *Cln1* and *Cln3* ko mice used in this study.[73] Indeed, in *Cln1* ko mice, atrophy in

certain anteroposterior brain regions was initially apparent at 3 months, stabilized by 5 months, and neurodegeneration was widespread by 7 months.[73] The stabilization observed at 5 months of age could represent the temporary efficacy of endogenous compensatory mechanisms that ultimately fail due to the presence of persistent pathological stressors as the animals succumb to disease by 7 months of age. Interestingly, the spatio-temporal patterns of metal accumulation in *Cln1* ko mice described here (Fig. 1-5) precede and for the most part predict the previously reported progressive regional brain atrophy. The brains of *Cln3* ko mice, however, displayed no reduction in brain volume until the age of 21 months, when degeneration was only observed in rostrocaudal region 3, which included the hippocampus and corticothalamic area,[73] although the cerebellum was not analysed in that study. Moreover, *Cln3* ko mice were reported to accumulate autofluorescent storage material in the retina, but this did not severely affect retinal function as assessed by electroretinogram testing in 11 month-old mice.[74] Similarly, although impaired motor function is observed from post-natal day 14 in these mice, rotarod scores did not progressively decline with time.[9] Thus, the subtle changes in metal content in *Cln3* ko mice compared to *Cln1* ko*, Cln5* ko and *Cln6 nclf* mice may reflect the mild rate of motor and visual symptom progression and late manifestation of brain atrophy in these animals. However, it is possible that examination of tissues from older mice may have yielded larger changes in metal content.

Subtle increases in CNS-wide metal concentrations in *Cln5* ko mice were detected from 5 months of age prior to vision loss, although lipofuscin deposition is already present. At 5 months, the most prominent increase was observed for cobalt followed by copper. By 7 months of age, all CNS regions had significantly elevated metal concentrations. It is noteworthy that the regional profile of metal elevation for most metals was consistent between 5 month-old *Cln1* ko and 7 month-old *Cln5* ko mice.

By contrast, in *Cln6 nclf* mice, tissue metal changes were first apparent in cortex and spinal cord, progressing to the cerebellum at a later presymptomatic age. By symptom onset at 8 months, biometal changes were evident in cortex and olfactory bulb, although the olfactory bulb was the only site of decreased metal content in *Cln6 nclf* mice at any age tested. In *Cln6 nclf* mice, the heart was also a site of progressive peripheral biometal accumulation, supportive of previously reported cardiac dysfunction in NCL patients.[75] In the models described here, however, the abnormalities in metal homeostasis appear to be confined to the CNS, at least at the ages tested.

The importance of appreciating the benefits and limitations of each NCL disease model studied cannot be understated. For example, *Cln3ΔEx1-6* mice used here have a mild disease phenotype, but tissue pathology that is reminiscent of JNCL patients,[8] while the *Cln3ΔEx7-8* mice display an aggressive form of disease progressing from abnormalities during the embryonic stage.[7] Similarly, *Cln1ΔEx9* mice lack progressive visual impairment,[4] compared to the *Cln1ΔEx4* mice used in this study.[3] *Cln6 nclf* mice carry a naturally occurring mutation in *Cln6*, whereas other NCL models are artificially generated. These considerations highlight the utility of examining multiple disease models as each may recapitulate particular disease features, but will not demonstrate all clinically relevant signs.

CONCLUSION

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2 We have previously demonstrated that deregulation of biometal homeostasis precedes
3 symptoms in 3 natural CLN6 sheep and mouse models, which may be caused by
4 impaired expression of the metal transporter Zip7. In the present study, we extended
5 these findings to include 3 additional models of NCL disease. We observed
6 progressive CNS-localized biometal accumulation, generally prior to symptom onset
7 and relative to the severity of disease in *Cln1, Cln3,* and *Cln5* mice, indicating that
8 metal homeostasis defects are a feature of at least 4 genetic forms of NCLs. Increased
9 expression mRNA encoding the metal-responsive anti-oxidant protein, Mt, verified
10 that perturbations to metal homeostasis are present in these models. Given that no
11 significant changes to metal transport proteins were detected, it is currently unclear
12 whether impaired biometal handling is an initiating factor contributing to
13 neurodegeneration, or a consequence of neurodegenerative changes in these mice.
14 Future studies will examine subcellular biometal handling to determine the
15 mechanistic changes underlying biometal accumulation in individual models of NCL
16 disease.

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19 **EXPERIMENTAL**

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21 **Mice**
22 This study used wild type (WT) Rcc mice, homozygous transgenic *Cln1* ko
23 (*Cln1ΔEx4*[3]) and *Cln5* ko mice (*Cln5ΔEx3*[12]), C57BL/6 WT and homozygous *Cln3*
24 ko mice (*Cln3ΔEx1-6*[8]) and WT and *Cln6 nclf* mice.[76] Animal handling and
25 experimentation were performed in accordance with national and institutional
26 guidelines (National Public Health Institute, University of Helsinki, King's College
27 London and Melbourne University, as well as State Provincial offices of Finland
28 (agreement numbers ESAVI/3474/04.10.03/2012, KEK12-017, and KTL 2005-02;
29 AEC# 1112024). The mice received a standard rodent diet and water, provided *ad*
30 *libitum*. The weights of all *Cln3* and *Cln5* ko mice were typical to that of WT
31 C57BL/6 mice (25-30g for 6-12 months), whereas *Cln1* ko mice progressively lose
32 weight and weigh on average 3-12g less than their WT counterparts. The genotypes of
33 affected *Cln1* ko*, Cln3* ko*, Cln5* ko and *Cln6 nclf* mice were determined as previously
34 described.[3, 8, 12, 31] Mice were euthanized by an intraperitoneal overdose of 2:1
35 Ketaminol vet (50 mg/ml, Intervet International, Netherlands) and Rompun vet
36 (xylaxin, 20 mg/ml, Bayer Health Care A-G, Germany; for *Cln1* and *Cln5* ko mice) or
37 an intraperitoneal overdose of Lethobarb (for *Cln3* ko mice) and were transcardially
38 perfused with 0.9% (w/v) saline containing 0.01% (w/v) heparin. At post mortem,
39 brains were dissected into the following regions for *Cln1* and *Cln5* ko mice (N=5-8
40 per genotype per age group, for male:female ratios and animal numbers, see
41 supplementary material, Table S1-S2): olfactory bulb, cortex, cerebellum, and brain
42 stem, and immediately frozen in liquid nitrogen. Spinal cords were also collected for
43 analysis. For *Cln3* ko mice (N=5 per genotype per age group), the brains were
44 dissected into: brainstem, cortex, cerebellum and thalamus. The spinal cord, heart and
45 liver were also collected and frozen for metal analyses. For *Cln6 nclf* mice (N=5 per
46 genotype), cortex, cerebellum and spinal cord were collected at post mortem.

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48 **ICP-MS**
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1 The metal contents in mouse brain and peripheral tissues were measured using
2 inductively coupled plasma mass-spectrometry (ICP-MS) as previously reported.[32]
3 Briefly, each CNS tissue piece from one hemisphere, or half of the peripheral tissue
4 for each mouse was weighed, lyophilized, digested in nitric acid overnight, and heated
5 at 90°C for 20 min. The acid treated samples were then treated with hydrogen
6 peroxide. After 30 min, the samples were heated for a further 15 min at 70°C. All
7 samples were diluted in 1 % nitric acid before being measured using an Agilent 7700
8 series ICPMS instrument using a Helium Reaction Gas Cell. The instrument was
9 calibrated using 0, 5, 10, 50, 100 and 500 ppb of certified multi-element ICPMS
10 standard calibration solutions (Accustandard, New Haven, CT, USA) for a range of
11 elements. 200 ppb of Yttrium (Y89) was used as an internal control (Accustandard).

qRT PCR

Due to limited amounts of tissue for each mouse cohort, we chose a primary
pathogenic tissue for investigation of *Mt* mRNA expression based on previous reports
of pathology in these mice, and a secondary tissue with which to perform western
blotting analyses. RNA was prepared and DNAse treated from 1-5mg of mouse
tissues using the MagMax Total RNA isolation kit (Life Technologies, Mulgrave,
Victoria, Australia) or Purelink RNA mini kit (Life Technologies). RNA (200ng) was
reverse transcribed using the High Capacity cDNA kit (Life Technologies). Mouse
endogenous control GAPDH (4352932) and TaqMan gene expression assays for
Mt1A, Mt2, Mt3 and Tuba8 were purchased from Life Technologies
(Mm00496660_g1, Mm00809556_s1, Mm0049666_g1 and Mm00833707_mH
respectively) and qRT-PCR was performed as previously described.[31] Delta Ct
method was used for normalisation of expression relative to β-tubulin or GAPDH.

Western Blotting

Cell lysates and tissues homogenized with a Dounce tissue grinder were extracted
with Phosphosafe (Merck, Kilsyth, Victoria, Australia) containing a protease inhibitor
cocktail (Roche, Castle Hill, NSW, Australia) and DNAse (Roche). Western blotting
was performed as previously described[31] using antibodies to the following zinc
transport proteins: Zip7 (1:2000, Proteintech, Chicago, IL, USA), Zip8 (1:1200,
Proteintech), Zip14 (Novus, Littleton, CO, USA), ZnT5 (1:1000, Abcam, Cambridge,
UK), ZnT6 (1:1200, Proteintech), ZnT7 (1:1000, Proteintech). Where visibly different
between control and transgenic animals (N≥5 animals per genotype), target band
intensities were compared to control bands (ImageJ, Bethesda, MD, USA) on blots
probed with antibodies against β-tubulin or total ERK used as controls to normalize
protein concentrations in affected and control animals.

Statistical Analysis
The effects of genotype and brain region on metal concentrations were examined
using 2-way ANOVA in GraphPad Prism software (GraphPad Software, La Jolla, CA,
USA). If either statistically significant main effects or interactions of brain region and
genotype were detected, the regions with differing metal concentrations were
individually determined using Bonferonni posttests. 1-way ANOVA was used to test
the effect of genotype on Mt mRNA levels.

ABBREVIATIONS

1	CNS	central nervous system
2	INCL	Infantile NCL
3	JNCL	Juvenile NCL
4	ko	knockout
5	LSD	lysosomal storage disorder
6	Mt	metallothionein
7	NCL	Neuronal Ceroid Lipofuscinosis
8	vLINCL	variant late infantile NCL

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3   **FIGURE LEGENDS**
4
5   Fig. 1. Zinc concentrations in the brain of *Cln1, Cln3* and *Cln5* ko mice. Zinc
6   concentrations in CNS of (A) 3 month-old, (B) 4 month-old, (C) 5 month-old or (D) 7
7   month-old WT mice, *Cln1* an *Cln5* ko mice were measured using ICP-MS. Zinc
8   concentrations in CNS of (E) 6 month-old and (F) 12 month-old WT mice and *Cln3*
9   ko mice were measured using ICP-MS. The concentrations of zinc in each tissue are
10   expressed as mean ± S.D. Values correspond to µg metal/g tissue. * $p<0.05$, **
11   $p<0.01$, *** $p<0.001$ by 2-way ANOVA and Bonferroni posttests. HC, hippocampus;
12   CX, cortex; CB, cerebellum; OB, olfactory bulb; SC, spinal cord; BS, brainstem; TH,
13   thalamus.

14   Fig. 2. Copper concentrations in the brain of *Cln1, Cln3* and *Cln5* ko mice. Copper
15   concentrations in CNS of (A) 3 month-old, (B) 4 month-old, (C) 5 month-old or (D) 7
16   month-old WT mice, *Cln1* and *Cln5* ko mice were measured using ICP-MS. Copper
17   concentrations in CNS of (E) 6 month-old and (F) 12 month-old WT mice and *Cln3*
18   ko mice were measured using ICP-MS. The concentrations of copper in each tissue
19   are expressed as mean ± S.D. Values correspond to µg metal/g tissue. * $p<0.05$, **
20   $p<0.01$, *** $p<0.001$ by 2-way ANOVA and Bonferroni posttests. Statistically
21   significant main effects of copper in WT compared with CLN3 brains were calculated
22   by 2-way ANOVA. HC, hippocampus; CX, cortex; CB, cerebellum; OB, olfactory
23   bulb; SC, spinal cord; BS, brainstem; TH, thalamus.

24   Fig. 3. Manganese concentrations in the brain of *Cln1, Cln3* and *Cln5* ko mice.
25   Manganese concentrations in CNS of (A) 3 month-old, (B) 4 month-old, (C) 5 month-
26   old or (D) 7 month-old WT mice, *Cln1* and *Cln5* ko mice were measured using ICP-
27   MS. Manganese concentrations in CNS of (E) 6 month-old and (F) 12 month-old WT
28   mice and *Cln3* ko mice were measured using ICP-MS. The concentrations of
29   manganese in each tissue are expressed as mean ± S.D. Values correspond to µg
30   metal/g tissue. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by 2-way ANOVA and Bonferroni
31   posttests. HC, hippocampus; CX, cortex; CB, cerebellum; OB, olfactory bulb; SC,
32   spinal cord; BS, brainstem; TH, thalamus.

34   Fig. 4. Iron concentrations in the brain of *Cln1, Cln3* and *Cln5* ko mice. Iron
35   concentrations in CNS of (A) 3 month-old, (B) 4 month-old, (C) 5 month-old or (D) 7
36   month-old WT mice, *Cln1* and *Cln5* ko mice were measured using ICP-MS. Iron
37   concentrations in CNS of (E) 6 month-old and (F) 12 month-old WT mice and *Cln3*
38   ko mice were measured using ICP-MS. The concentrations of iron in each tissue are
39   expressed as mean ± S.D. Values correspond to µg metal/g tissue. * $p<0.05$, **
40   $p<0.01$, *** $p<0.001$ by 2-way ANOVA and Bonferroni posttests. Statistically
41   significant main effects of iron in WT compared with CLN3 brains were calculated by
42   2-way ANOVA. HC, hippocampus; CX, cortex; CB, cerebellum; OB, olfactory bulb;
43   SC, spinal cord; BS, brainstem; TH, thalamus.

45   Fig. 5. Cobalt concentrations in the brain of *Cln1, Cln3* and *Cln5* ko mice. Cobalt
46   concentrations in CNS of (A) 3 month-old, (B) 4 month-old, (C) 5 month-old or (D) 7
47   month-old WT mice, *Cln1* and *Cln5* ko mice were measured using ICP-MS. Cobalt
48   concentrations in CNS of (E) 6 month-old and (F) 12 month-old WT mice and *Cln3*
49   ko mice were measured using ICP-MS. Cobalt levels were below detection limits in
50   the thalamus of WT and *Cln3* ko mice. The concentrations of cobalt in each tissue are

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1 expressed as mean ± S.D. Values correspond to μg metal/g tissue. * *p*<0.05, **
2 *p*<0.01, *** *p*<0.001 by 2-way ANOVA and Bonferroni posttests. Statistically
3 significant main effects of cobalt in WT compared with CLN3 brains were calculated
4 by 2-way ANOVA. HC, hippocampus; CX, cortex; CB, cerebellum; OB, olfactory
5 bulb; SC, spinal cord; BS, brainstem; TH, thalamus.
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7 Fig. 6. Metallothionein mRNA induction in NCL mutant mice. Metallothionein (*Mt1,*
8 *2* or *3*) mRNA expression in was measured using qRT-PCR in (A) the cortex of 5
9 month-old WT, *Cln1* and *Cln5* ko mice, (B) the cerebellum of 6 month-old WT or
10 *Cln3* ko mice, and (C-E) the cerebellum, cortex and spinal cords of 3 month-old *Cln6*
11 *nclf* mice. Expression values were normalized to tubulin or GAPDH using the delta Ct
12 method. Data are expressed as fold Mt mRNA compared to WT controls. * *p*<0.05,
13 ** *p*<0.01, *** *p*<0.001 by 1-way or 2-way ANOVA and Bonferroni posttests, as
14 appropriate.
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16 Fig. 7. Biometal trafficking pathways in the brains of *Cln1, Cln3* and *Cln5* ko mice.
17 Representative immunoblots of homogenates (5-40 μg) isolated from the cerebellum
18 of 7 month-old WT and *Cln5* ko mice, 5 month-old WT and *Cln1* ko mice or the
19 cortex of 6 month-old WT and *Cln3* ko mice (minimum N=5 animals per group)
20 probed with antibodies directed against a range of metal transporter proteins of the
21 Zip and ZnT families. β-tubulin was used as loading controls for *Cln1* and *Cln5* ko
22 mice, whereas total ERK was used as a control for *Cln3* ko mice. N.D. no protein
23 detected.
24
25 **TABLES**
26
27 Table 1. Characteristics of mouse models analysed.
28 ^ motor dysfunction in not progressive
29 * HC, hippocampus; CX, cortex; CB, cerebellum; OB, olfactory bulb; SC, spinal
30 cord; BS, brainstem; TH, thalamus.

Disease	Model	Visual/motor symptoms	Storage material	Lifespan	Ages analysed	Tissues analysed*
INCL	*Cln1ΔEx4* [3]	3.5/4m[3]	3m[3]	6.5m[3]	3,4,5m	HC, CX, CB, OB, SC
vLINCL	*Cln5ΔEx3* [12]	5.2m/>8m[12]	3m[12]	>8m[34]	3,4,5,7m	HC, CX, CB, OB, SC
vLINCL	*Cln6 nclf* [76]	4/8m[76]	P11[76]	12m[76]	3m	CX, CB, SC
JNCL	*Cln3ΔEx1-6* [8]	>11m[74]/E14[9^]	5m[8]	>21m[73]	6,12m	BS, CX, TH, SC, CB

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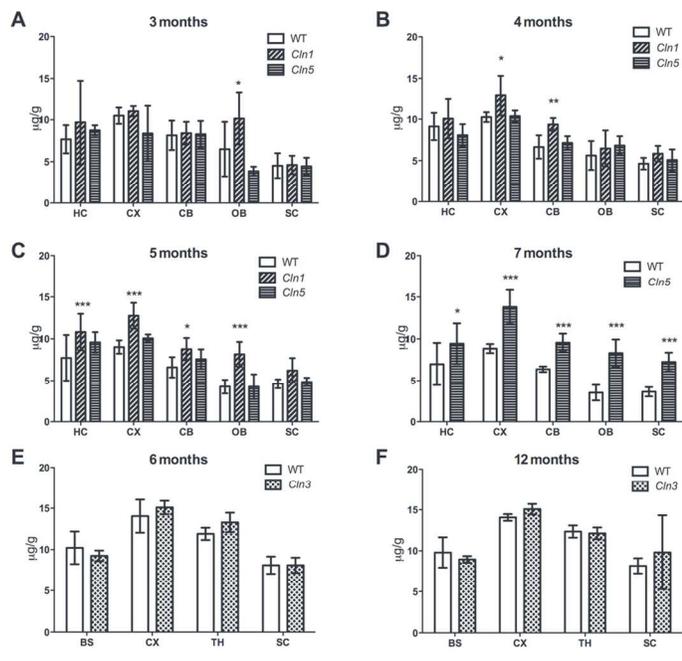
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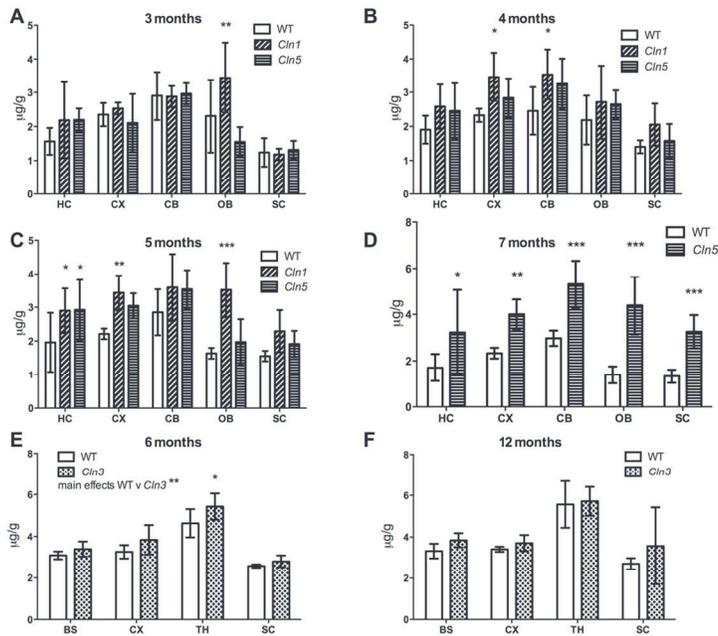
117x80mm (300 x 300 DPI)
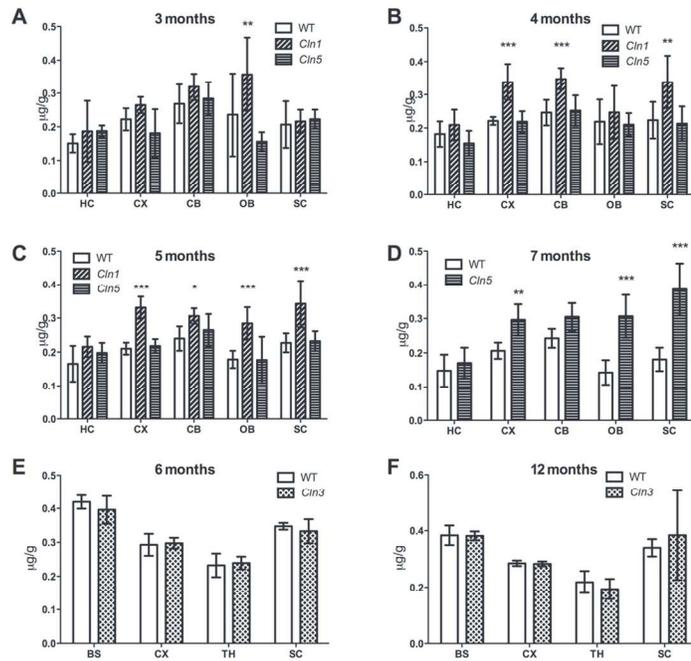
122x87mm (300 x 300 DPI)

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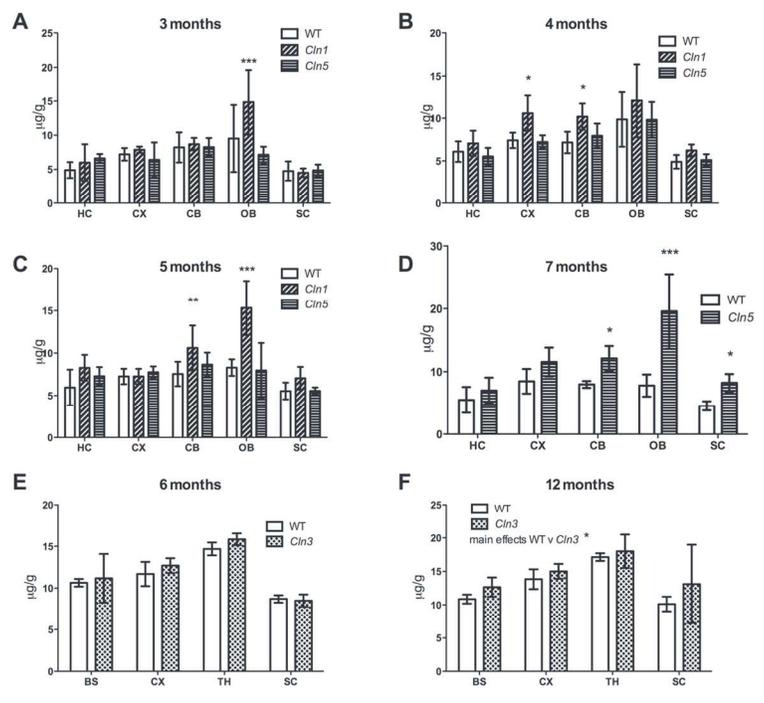
126x93mm (300 x 300 DPI)

167x163mm (300 x 300 DPI)

203x241mm (300 x 300 DPI)

	WT	*Cln5*	WT	*Cln1*	WT	*Cln3*
Zip7						
Zip8						
Zip14			N.D.			
ZnT5						
ZnT6			N.D.			
ZnT7			N.D.			
Loading						

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