Exacerbated neuronal ceroid lipofuscinosis phenotype in Cln1/5 double knock-out mice

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SUMMARY

Both *CLN1* and *CLN5* deficiency leads to severe neurodegenerative diseases of childhood, known as neuronal ceroid lipofuscinoses (NCL). The broadly similar phenotypes of NCL mouse models, and the potential for interactions between NCL proteins, raise the possibility of shared or convergent disease mechanisms. To begin addressing these issues we have developed a novel mouse model lacking both *Cln1* and *Cln5* genes. These *Cln1/5* double knock-out (*Cln1/5 dko*) mice were fertile, showing a slight decrease in expected Mendelian breeding ratios, as well as impaired embryoid body formation by induced pluripotent stem cells derived from *Cln1/5 dko* fibroblasts. Typical disease manifestations of the NCLs, seizures and motor dysfunction, were detected at the age of 3 months, earlier than in either single knock-out mouse. Pathological analyses revealed a similar exacerbation and earlier onset of disease in *Cln1/5 dko* mice, which exhibited a pronounced accumulation of autofluorescent storage material. Cortical demyelination and more pronounced glial activation in cortical and thalamic regions was followed by cortical neuron loss. Alterations in lipid metabolism in *Cln1/5 dko* showed a specific increase in plasma phospholipid transfer protein (PLTP) activity. Finally, gene expression profiling of *Cln1/5 dko* cortex revealed defects in myelination and immune response pathways, with a prominent downregulation of α-synuclein in *Cln1/5 dko* mouse brains. The simultaneous loss of both *Cln1* and *Cln5* genes may enhance the typical pathological phenotypes of these mice by disrupting down shared or convergent pathogenic pathways, which may potentially include interactions of CLN1 and CLN5.

**Keywords:** CLN1; CLN5; NCL; thalamocortical degeneration; glial activation; gene expression profiling
INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) represent the most frequent group of inherited neurodegenerative diseases in children, with an estimated worldwide incidence of 1:14,000-1:67,000, depending on ethnic group and founder group effect (Santavuori, 1988; Haltia, 2006; Mole et al., 2011). The NCLs are characterized by progressive visual and mental decline, motor disturbances, epilepsy and behavioral changes, ultimately leading to premature death. These autosomal recessive diseases are caused by mutations in at least ten genes, including eight conventional NCL-associated genes (CLN1, CLN2, CLN3, CLN5, CLN6, CLN7/MFSD8, CLN8, and CTSD/CLN10), and two additional genes (DNAJC5 and SGSH) (Kousi et al., 2012). Despite this genetic heterogeneity, the NCLs display a relatively uniform phenotype in the central nervous system (CNS), characterized by a dramatic loss of cortical neurons, and accumulation of lysosomal autofluorescent lipopigments (Jalanko and Braulke, 2009; Mole et al., 2011).

Here, we focus on two NCL diseases, CLN1, classic infantile NCL and CLN5, late infantile variant NCL, which are caused by the mutations in CLN1 and CLN5 genes, respectively (Vesa et al., 1995; Savukoski et al., 1998; Kousi et al., 2012). CLN1 encodes for PPT1, a soluble lysosomal enzyme that is involved in depalmitoylation of proteins (Camp and Hofmann, 1993), although the in vivo substrates of PPT1 are currently unknown (Hellsten et al., 1995; Jalanko and Braulke, 2009). The CLN5 gene encodes a soluble lysosomal glycoprotein, implicated recently in endosomal sorting (Schmiedt et al., 2010; Mamo et al., 2012). CLN5 protein co-localizes with lysosomal associated membrane protein (LAMP-1) (Vesa et al., 2002; Schmiedt et al., 2010), and is expressed by both neurons and glia (Holmberg et al., 2004; Schmiedt et al., 2012).

We have previously generated Cln1 and Cln5 knock-out (ko) mouse models (Jalanko et al., 2005; Kopra et al., 2004). The Cln1 ko mouse model presents with a severe neurodegenerative
disease, whereas Cln5 ko mice have a much milder phenotype that progresses more slowly. Both of these mice suffer from several neurological defects, including motor dysfunction, and exhibit progressive weight loss and brain atrophy. Neuropathological analyses show progressive accumulation of autofluorescent storage material, and a marked glial activation, which precedes neuronal loss, and is most pronounced in the thalamocortical system. Gene expression profiling of cortical tissue revealed common affected pathways in Cln1 ko and Cln5 ko mouse models, including inflammation, cytoskeleton integrity, and the neuronal growth cone assembly (von Schantz et al., 2008). Molecular interaction studies of NCL proteins have shown that CLN5 appears to interact with other NCL proteins in vitro, including CLN1/PPT1, CLN2 (tripeptidyl-peptidase 1, TPP1), CLN3, CLN6 and CLN8 (Vesa et al., 2002; Lyly et al., 2009). In addition, CLN1 and CLN5 share a common interaction partner, the F1 subunit of the ATP-synthase, which modulates the lipid homeostasis in neurons (Lyly et al., 2008, 2009).

The finding that similar functional pathways are affected in these forms of NCL and the potential interaction of CLNs at the protein level, raise the possibility that NCL diseases may also be linked at a molecular level. To gain a deeper insight into the shared molecular and pathological events behind the NCLs and the underlying mechanisms of neurodegeneration, we developed a Cln1/5 double knock-out (Cln1/5 dko) mouse model. These mice were first analyzed for the possible defects in embryonic development using induced pluripotent stem (iPS) cell technology. In the brain the accumulation of autofluorescence storage material, presence of astrocytosis, microglial activation and signs of myelination defects were also investigated and stereological analyses were performed to study brain atrophy and neuronal loss. This analysis focused upon the onset and progression of thalamic and cortical neuron loss, based on the changes we have
documented in mice singly deficient for either Cln1 or Cln5 (Kopra et al., 2004; Jalanko et al., 2005).

Since previous studies have suggested that CLN1 and CLN5 proteins and diseases are closely related and linked to changes in cellular lipid homeostasis (Lyly et al., 2008; Schmiedt et al., 2012), we also conducted plasma lipid profiling of Cln1/5 dko mice. Finally, gene expression profiling of the Cln1/5 dko mouse cortex was performed in order to reveal the functional pathways that may be shared by CLN1 and CLN5 diseases.

RESULTS

Cln1/5 dko iPS cells show impaired embryoid body (EB) formation and delayed differentiation potential

The Cln1/5 dko offspring were viable and fertile. However, the cross-breeding of Cln1 ko and Cln5 ko mice produced fewer double homozygous Cln1/5 dko offspring than expected according to Mendelian ratios (Table S1), when the parents were homozygous for one of the alleles and heterozygous or homozygous for the other allele. To study a possible defect in breeding or early development of Cln1/5 dko mice in more detail we generated several iPS cell lines from Cln1/5 dko and wt MEFs. Two wt (clones15 and 23) and three different Cln1/5 dko (clones 3, 19-2 and 20) iPS cell clones were selected for further experiments on the basis of their positive expression of ES cell markers SSEA-1, Nanog, Rex-1, Fgf-4, Esg-1 and E-ras (Fig. S1) and their differentiation potential into embryonic germ layers (Fig. S2). Equal numbers of feeder-free cells from wt and Cln1/5 dko iPS clones were plated in parallel for EB formation and the experiment was repeated three times with different clones. Daily microscopic analyses of growing EBs showed that between days two and seven the Cln1/5 dko EBs were consistently smaller and more
irregularly shaped than the wt EBs (Fig. 1A, an example of one experiment). Furthermore, the total number of EBs formed from Cln1/5 dko iPS cell clones appeared to be smaller compared to those from wt clones. Calculation of the diameter of seven day old EBs in different clones revealed that this reduction in EB size was also statistically significant (Fig. 1B). This finding was consistent across three different Cln1/5 dko iPS cell clones. From day seven onwards, both the size and the shape of EBs became more similar between the Cln1/5 dko and wt, indicating problems in early development of EBs (data not shown). The formed EBs were then plated on gelatin-coated plates to allow spontaneous differentiation and cell outgrowth from EBs. Microscopic analyses showed that cellular outgrowth from Cln1/5 dko EBs was much slower than from the wt EBs. On day three after plating, wt EBs had lost their round shape and cellular growth was seen all over the plates. In contrast, Cln1/5 dko EBs were still round and cellular growth was only detected close to the EBs (Fig. 1C), indicating that spontaneous cell differentiation was also delayed. These possible defects in early development could explain the detected decrease in the number of the born offspring of Cln1/5 dko mice.

**Cln1/5 dko mice display an early neuropathological phenotype**

The Cln1/5 dko mice presented with a rapidly progressing NCL disease phenotype, already displaying severe symptoms, including seizures and motor dysfunction, by the age of 3 months, much earlier than Cln1 ko mice (5 months) or Cln5 ko mice (8 months) (Jalanko et al., 2005; Kopra et al., 2004) (data not shown). The onset of the symptoms and the progression of the disease were slightly variable among the Cln1/5 dko mice. All three mouse models showed a significant reduction in body weight at the age of 3 months, compared to wt controls (Fig. S3). Cln1/5 dko mice were euthanized at the age of 3 months due to their severe disease phenotype.
The accumulation of autofluorescent lipopigments is a key pathological feature of all forms of NCL and was analyzed in Cln1 ko, Cln5 ko, Cln1/5 dko and wt control mice via confocal microscopy. Increased amounts of punctate autofluorescent material were observed in the thalamus and cortex of 3 month old Cln1 ko, whereas in Cln5 ko and in wt controls it was almost absent at this age. In contrast, Cln1/5 dko mice displayed a much more pronounced accumulation of autofluorescent material (Fig. 2A), and this was already seen in 1 month old Cln1/5 dko mice (data not shown). Thresholding image analysis showed elevated, but not significant levels of autofluorescent material in the cortex and thalamus of Cln1 ko and Cln1/5 dko mice, compared to wt (Fig. 2B).

The ultrastructural appearance of the storage material is specific to individual forms of NCL and it differs between these forms (Haltia, 2003; Tyynelä et al., 1993; Tyynelä et al., 2004). On a mixed C57BL background, Cln1 ko mice show accumulation of granular osmiophilic deposits (GRODs) in the brain and other tissues, whereas in Cln5 ko mice, the storage material displays rectilinear, curvilinear and fingerprint profiles (Jalanko et al., 2005; Kopra et al., 2004). In C57BL/6JRcc background, the ultrastructure of the storage material in Cln1 ko mice was composed of GRODs, and in Cln5 ko the storage material varied from rectilinear to fingerprint-like (Fig. 2C.5 and 2C.6). Electron microscopic analysis of 3 month old Cln1/5 dko mouse brains revealed marked accumulation of GRODs within cortical and thalamic neurons (Fig. 2C.1, 2C.3). The ultrastructure of the storage deposits was almost identical to that seen in Cln1 ko mice (Fig. 2C.5), but the storage deposits were much more abundant in the double ko mice compared to either of the single ko mice. Within cortical neurons, tiny electron dense grains were visible inside these GRODs (Fig. 2C.2), while in the thalamus, the storage bodies appeared to be more
electron dense than in the cerebral cortex (Fig. 2C.3, 2C.4). Higher magnification shows that the GRODs are surrounded by a double membrane (Fig. 2C.2, 2C.4).

**Early immune response is enhanced in Cln1/5 dko mouse**

Localized astrocytosis and microglial activation has been reported to occur both in Cln1 ko and Cln5 ko mice (Bible et al., 2004; von Schantz et al., 2009; Schmiedt et al., 2012). To investigate the extent of astrocytosis in Cln1/5 dko mice we stained brain sections from 3 month old mice for the astrocytic marker GFAP, and compared them to Cln5 ko, Cln1 ko and wt mice at the same age. We concentrated our analysis upon primary somatosensory barrelfield cortex (S1BF) and thalamic ventral posteromedial nucleus/ventral posterolateral nucleus (VPM/VPL), which relays sensory information to this cortical subfield, since both of these structures are consistently severely affected in NCL mouse models (Cooper, 2010). As previously reported (von Schantz et al., 2009), only few scattered GFAP positive astrocytes were present in VPM/VPL of young Cln5 ko mice, but many more intensely stained astrocytes were present in this thalamic nucleus of young Cln1 ko mice. However, GFAP staining of the corresponding brain region in Cln1/5 dko mice revealed a more profound astrocytosis, significantly greater than that seen in Cln5 ko mice and modestly increased compared to Cln1 ko mice with the VPM/VPL and surrounding nuclei of Cln1/5 dko mice completely filled with hypertrophic and intensely GFAP-immunoreactive astrocytes (Fig. 3A). Comparing single and double mutant mice revealed a similar relationship in degree of staining in the S1BF region of the cortex, showing pronounced astrocytosis all across cortical laminae in Cln1 ko mice, but with additional bands of more intensely and darkly stained astrocytes in laminae II-IV and VI in Cln1/5 dko mice (Fig. 3A). Thresholding image analysis confirmed the significant increase in GFAP immunoreactivity in
VPM/VPL and S1BF regions of Cln1 ko and Cln1/5 dko brains, compared to the wt brains (Fig. 3C).

Activated microglia can be detected by the expression of CD68, a lysosomal/endosomal membrane glycoprotein (Holness et al., 1993). In wt mice palely stained CD68 positive microglia were present in most CNS regions, but at 3 months of age widespread CD68 immunoreactivity was present in the brains of all three mutant mice, most notably in the thalamic relay nuclei, lateral and medial geniculate nuclei, cortical regions (S1BF), the subiculum and selected hippocampal subfields, the globus pallidus, and substantia nigra. Focusing upon the S1BF and VPM/VPL regions, microglial activation was consistently more advanced in this thalamic relay nucleus than in its corresponding cortical target region (Fig. 3B). Consistent with our recent findings (Schmiedt et al., 2012), 3 month old Cln5 ko mice already showed evidence of microglial activation with CD68 stained microglia displaying larger and more darkly stained cell soma than in wt controls. This phenotype was more pronounced in Cln1 ko mice with a subset of microglia displaying a brain-macrophage-like morphology, but was exacerbated further in Cln1/5 dko mice in which the entire thalamus was filled with intensely stained CD68 positive microglia with swollen cell soma and only very short cell processes (Fig. 3B). An elevated level of CD68 staining was already detectable in both thalamus and cortex of Cln1/5 dko mice at 1 month of age (Fig. S4). Thresholding image analysis verified this widespread and significant microglial activation in the S1BF of Cln1/5 dko mouse brains, compared to wt mice (Fig. 3D). Due to a higher degree of variation, this elevated CD68 expression did not exceed the significant levels of microglial activation in the VPM/VPL or S1BF regions of Cln5 ko, that we have previously reported (Schmiedt et al., 2012).
Disturbed cortical myelination in *Cln1/5* dko mouse

Disturbed myelination has previously been described in *Cln5* ko mice (Kopra et al., 2004; von Schantz et al., 2009; Schmiedt et al., 2012), but has not been studied in *Cln1* ko mice. Immunostaining for MBP, a main protein constituent of the myelin sheath, revealed no overt disruption of white matter structures in 1 and 3 month old *Cln5* ko mice, but the superficial laminae of S1BF contained fewer MBP positive fibers (Schmiedt et al., 2012). Performing similar analyses in this study revealed more pronounced effects on MBP staining in this brain region of *Cln1/5* dko mice, compared to either *Cln1* ko, or *Cln5* ko mice. Compared to wt controls, 1 month old *Cln1/5* dko mice displayed fewer MBP immunopositive fibers running dorsoventrally and horizontally through the superficial laminae (laminae II and III) of S1BF (Fig. 4A). By 3 months of age all three mutant mouse models showed fewer MBP positive fibers in the superficial laminae of S1BF compared to wt controls (Fig. 4B).

*Cln1/5* dko mouse shows neuron loss in cortical lamina VI at 3 months of age

Cortical atrophy is a common feature of all human and murine NCLs (Cooper et al., 2006), but occurs relatively late in disease progression. In order to determine if this phenotype was already apparent in 3 month old *Cln1/5* dko mice, we carried out thickness measurements in Nissl stained sections through primary motor (M1), S1BF, primary visual (V1) and lateral entorhinal (LEnt), as representative cortical regions that serve different functions. Compared to wt controls, no significant thinning was observed in any cortical region of 3 month old *Cln1/5* dko brains, or in age-matched *Cln1* ko mice (Fig. S5A). As reported previously (von Schantz et al., 2009), the cortical thickness of S1BF, M1 and V1 in *Cln5* ko mice was significantly increased at this age.
We next conducted a stereological analysis of regional volumes in *Cln1* ko, *Cln5* ko, *Cln1/5* dko and wt brains at 3 months of age. Cavalieri estimates of regional volumes revealed no significant atrophy of the cortex or hippocampus of the *Cln1/5* dko mice at this age. Consistent with the thickness measurements, the cortical volumes of *Cln5* ko mice were significantly increased compared to the wt controls. In addition, the hippocampal volume was significantly increased in the *Cln5* ko brain (Fig. S5B).

To investigate the extent of neuron loss in *Cln1/5* dko mice, we conducted optical fractionator estimates of the number of Nissl stained VPM/VPL neurons, and of neuron populations in three cortical laminae of S1BF; lamina IV granule neurons that receive thalamic innervations, projection neurons in lamina V, and lamina VI neurons that supply feedback to the thalamus. We chose these regions since they are characteristically vulnerable in mouse models of NCL (Pontikis et al., 2005; Weimer et al., 2006; Kielar et al., 2007; Partanen et al., 2008). This analysis revealed consistently fewer neurons in all brain regions of 3 month old *Cln1/5* dko mice, than in mice deficient for either *Cln1* or *Cln5* alone. However, it was only in lamina VI of S1BF of 3 month old *Cln1/5* dko mice that this neuron loss was statistically significant (Fig. 5A-D).

**Lipid profiles in *Cln1/5* dko mouse plasma show significant increase in phospholipids and PLTP activity**

Alterations in systemic lipid metabolism have been reported in both *Cln1* ko (Lyly et al., 2008) and *Cln5* ko mice (Schmiedt et al., 2012). Therefore, we wanted to assess possible lipid metabolism changes when both *Cln1* and *Cln5* are deleted. Plasma cholesterol, phospholipids, triglycerides, apoA-1 and the activity of PLTP in 1 month old female mice were analyzed from wt, *Cln5* ko, *Cln1* ko and *Cln1/5* dko mice. Phospholipid levels in *Cln1/5* dko mice were
significantly increased, whereas cholesterol, triglyceride and apoA-1 levels were slightly elevated compared to wt controls (Fig. 6A). Total cholesterol levels were significantly decreased in Cln5 ko plasma, as well as in Cln1 ko plasma, as reported previously (Lyly et al., 2008). However, in this study, the cholesterol levels in Cln5 ko mice did not significantly differ from those of wt mice, as previously reported (Schmiedt et al., 2012). Increased plasma PLTP activity is suggested to be associated with increased cholesterol levels (Colhoun et al., 2001), and PLTP activity was indeed significantly increased in Cln1/5 dko mice compared to wt mice (Fig. 6B). However, plasma PLTP activity in Cln1 ko and Cln5 ko mice did not differ from that of wt controls and therefore did not support our previous results (Lyly et al., 2008; Schmiedt et al., 2012).

To verify the detected lipid changes in Cln1/5 dko mice we also analyzed the distribution of lipids and apoA-1 in lipoprotein fractions. Cholesterol levels in were clearly elevated in high density lipoprotein (HDL) particles, and slightly decreased in very low density lipoprotein (VLDL) particles of Cln1/5 dko plasma (Fig. S6A). In addition, the elevated PLTP activity led to enhanced phospholipid transfer from the VLDL pool to HDL pool (Fig. S6A). ApoA-1 was clearly elevated in both small (pre-β-HDL) and large HDL particles (Fig. S6B), indicating increased PLTP activity, which is known to induce the formation of lipid-poor pre-β-HDL particles (Siggins et al., 2007). Furthermore, elevated triglyceride levels were found in VLDL particles (Fig. S6C). Together, these results suggest a change in systemic lipid homeostasis in Cln1/5 dko mice.
Gene expression profiling of the Cln1/5 dko mouse cortex

Global gene expression analysis of approximately 45,000 transcripts from 1 month old Cln1/5 dko cortex revealed statistically significant upregulation of 24 genes and downregulation of 31 genes (Table 1). The most upregulated gene was cyclase-associated protein 1 (Cap1, 1.9-fold), and the most downregulated gene was α-synuclein (Snca, -177.2-fold). Other upregulated genes included complement subcomponents C1qb (C1qb, 1.6-fold) and C1qc (C1qc, 1.4-fold), Fc receptor homolog S (Fcrls, 1.6-fold) and glial fibrillary acidic protein (GFAP, 1.5-fold). Downregulation of oligodendrocytic myelin and paranodal and inner loop protein (Opalin, -1.8-fold), as well as other myelin genes (myelin and lymphocyte protein Mal, -1.6-fold, myelin associated glycoprotein Mag, -1.6-fold, myelin oligodendrocyte glycoprotein Mog, -1.5-fold, proteolipid protein Plp1, -1.2-fold and myelin basic protein Mbp, -1.2-fold) was also observed. The Ppt1 (Cln1) gene was also downregulated (-5.1-fold), whereas downregulation of the Cln5 gene was not observed, due to the lack of a specific probe in the gene expression array.

Analyses of biological pathways are often more informative than single gene changes. In order to characterize the affected pathways in 1 month old Cln1/5 dko mice, and to compare them to the existing data from Cln1 ko and Cln5 ko mice, we performed a DAVID Functional Annotation Clustering. We discovered that pathways related to immune response were upregulated, whereas pathways related to myelin ensheathment and transmission of nerve impulse were downregulated (Table 2). These results are in line with the published defects in nerve ensheathment and myelination, and upregulation of inflammation associated pathways in Cln1 ko mice and neurons (Ahtiainen et al., 2007; von Schantz et al., 2008). A common gene upregulated in all three models (Cln1 ko, Cln5 ko and Cln1/5 dko) is Cap1, a gene critical for the neuronal growth cone-cytoskeletal dynamics (Ahtiainen et al., 2007; von Schantz et al., 2008; this study). Our results
from immunohistochemical studies indicate enhanced glial activation and defective myelination in \textit{Cln1/5} dko brain, and are thus consistent with the gene expression analysis data.

The downregulation of the most differentially expressed gene between the two genotypes (wt and \textit{Cln1/5} dko), \textit{Snca}, was further evaluated with quantitative real-time RT-PCR assay. The results support the microarray data, demonstrating almost complete lack of \textit{Snca} expression in the \textit{Cln1/5} dko cortex (Fig. S7A).

**Decreased \(\alpha\)-synuclein expression in \textit{Cln1/5} dko mouse cortex**

As a novel finding, gene expression profiling indicated a pronounced downregulation of \(\alpha\)-synuclein gene expression in the \textit{Cln1/5} dko mouse cortex. To validate this result at the protein level, we performed Western blotting of cortical and cerebrum (whole brain apart from the cerebellum) lysates from 1 and 3 month old mice. In cortical lysates from 3 month old \textit{Cln1/5} dko mice, the expression of full-length (19 kDa) \(\alpha\)-synuclein was completely absent compared to wt controls (Fig. 7A). In the cerebrum, the expression of \(\alpha\)-synuclein protein was absent in three out of five \textit{Cln1/5} dko lysates, while two out of five had normal \(\alpha\)-synuclein levels (Fig. 7B).

Similarly, no expression of \(\alpha\)-synuclein was observed in the 1 month old \textit{Cln1/5} dko mouse cortex (Fig. S7B), and expression was reduced in the cerebrum of \textit{Cln1/5} dko mice (Fig. S7B). Quantification of \(\alpha\)-synuclein expression levels showed significant reduction in \(\alpha\)-synuclein expression in both cortex and cerebrum of 1 (Fig. S7D) and 3 (Fig. 7C) month old \textit{Cln1/5} dko mice compared to wt controls. The cerebrum of 1 and 3 month old \textit{Cln1} ko and \textit{Cln5} ko mice expressed unchanged \(\alpha\)-synuclein levels (Fig. S8).

\(\alpha\)-Synuclein protein expression levels were also investigated by immunohistochemical staining in 3 month old wt, \textit{Cln1} ko, \textit{Cln5} ko and \textit{Cln1/5} dko brain sections (Fig. 7D). We observed
markedly reduced α-synuclein staining in the cortical neuropil, as well as some reduction in the hippocampus of Cln1/5 dko mice. In Cln5 ko mice, cortical and hippocampal α-synuclein immunoreactivity was also somewhat reduced. These results support our Western blot findings that show weak α-synuclein expression in the cortex, and close to normal expression levels in the cerebrum of a subset of Cln1/5 dko mice.

DISCUSSION
Characterization of NCL mouse models is essential for obtaining new information about disease progression and for a better understanding of the mechanisms behind the disease. In this study, we have created a novel Cln1/5 dko mouse model, and provide a detailed description of neuropathological events, focusing on the consequences of a deficiency in both of these genes. In general, the Cln1/5 dko mice exhibit an earlier onset and more severe neurological and neuropathological phenotypes than was seen in the individual Cln1 ko or Cln5 ko mouse models (summarized in Table 3).

Possible developmental defects are associated with Cln1/5 deficiency
Despite their severe neurological phenotype, the Cln1/5 dko mice are viable, but produce fewer double homozygous offspring than expected according to Mendelian ratios, probably due to reduced fertility. Furthermore, our data show that EB formation of Cln1/5 dko mice iPS cells is impaired, and further differentiation of EBs is much slower compared to that of wt controls. This could indicate that simultaneous deficiency of Cln1 and Cln5 genes may impact fetal survival in mice, seen as a lower number of offspring. Indeed, similar findings have previously been shown to be specifically associated with the iPS cells derived from another lysosomal storage disease,
mucopolysaccharidosis VII (Meng et al., 2010). Homozygous mutations of both Cln1 and Cln5 genes in human patients have not been reported, but CLN1 patients with homozygous mutation of Cln1 gene accompanied with an extra heterozygous mutation in Cln5 gene are known (Kousi, 2012). Our results indicate that the simultaneous dysfunction of both Cln1 and Cln5 genes worsens the clinical picture in mice, and in vitro experiments also suggest partial lethality. Considering these data, it is possible that the homozygous mutations in both CLN1 and CLN5 genes may be lethal in humans and therefore may not be detected in analyses of NCL disease causing mutations.

**Glial activation and defective myelination precede neurodegeneration in Cln1/5 dko brain**

Astrocytosis has long been considered a hallmark of neurodegenerative changes. Significant upregulation of GFAP is evident in immunohistochemistry and microarray studies of Cln1 ko and Cln5 ko mice (Elshatory et al., 2003; von Schantz et al., 2008, 2009). Our current data are consistent with these findings, with increased levels of astrocytosis in every region of the Cln1/5 dko mouse CNS, which is already strong at the age of 3 months. Microglial activation was also widespread in 3 month old Cln1/5 dko CNS, and was especially pronounced in the thalamocortical system. Microglia are the brain’s intrinsic immune cells, which play important roles in injury and neurodegenerative diseases (Perry et al., 2010). Microglia serve as damage sensors, and injury or pathological process leads to activation of these cells from their resting state. Thus, this microglial activation in Cln1/5 dko might be a response to increased cellular damage caused by storage material accumulation and lysosomal enlargement.

Reactive astrocytes and microglia often accompany neuronal loss and may be indicative of neuronal dysfunction (Gehrmann et al., 1995; Macauley et al., 2011). Indeed, in mouse and large
animal models of NCL glial activation invariably precedes neuron loss and is the most accurate predictor of where neurons are subsequently lost (Cooper, 2012). In this respect, the more enhanced reactive phenotype of Cln1/5 dko mice suggests that neuron loss would also be more pronounced if these mice were allowed, or able, to survive longer. Our investigation of neuron survival in these mice does reveal an earlier onset of neuron loss, although this did not reach significance in most brain regions. Nevertheless, it is evident that deficiency of both Cln1 and Cln5 clearly exacerbates this characteristic and early neuropathological feature of NCL disease progression.

Neuron loss begins in cortical lamina VI in Cln1/5 dko mice

In the Cln1 ko mouse model, neuron loss begins in the thalamus and only subsequently occurs in the corresponding cortical region (von Schantz et al., 2009). In contrast, in Cln5 ko mice, neuron loss begins in the cortex and only subsequently occurs in thalamus (von Schantz et al., 2009). In Cln1/5 dko, the only significant neuron loss was observed in cortical lamina VI of S1BF, suggesting that as in Cln5 ko mice, neuron loss in the thalamocortical system of Cln1/5 dko mice also occurs first in the cortex at the age of 3 months of age. It is perhaps surprising that more pronounced neuron loss was not seen in these Cln1/5 dko mice, but it should be remembered that this is the earliest documented incidence of significant neuron loss in one of these mouse models and has already occurred at a relatively young age. It is likely that neuron loss would become far more pronounced if these mice were to survive longer, especially given the profound nature of the exacerbated glial response they display.

In our study, no significant thinning of Cln1/5 cortex was seen at the age of 3 months. However, Cln5 ko mice did display a significantly increased thickness in the S1BF, M1 and V1 at this age.
Similar findings have been reported previously by von Schantz et al. (2009), showing increased thickness in the superficial laminae I-III of 12 month old Cln5 ko mice. Similarly, regional volume measurements revealed no atrophy in Cln1/5 dko brain at the age of 3 months, whereas increased volumes were observed in 3 month old Cln5 ko cortex and hippocampus.

**Altered lipid and lipoprotein plasma profile in Cln1/5 dko mice**

Previous studies have shown that cholesterol metabolism is disturbed in Cln1 ko mice (Ahtiainen et al., 2007; Lyly et al., 2008), and more recent studies have indicated even more prominent aberrations in the systemic lipid homeostasis of Cln5 ko mice (Schmiedt et al., 2012). In this study, we conducted plasma lipid profiling of Cln1/5 dko mice and observed significantly elevated levels of phospholipids and increased PLTP activity in these mice. These lipid changes were accompanied by the differential distribution of phospholipids and cholesterol in HDL particles. Although the previously published results of plasma lipid analyses of single ko mice were not completely replicated in this study, it is clear that both Cln1 and Cln5 ko mice show changes in lipid metabolism. Embryonic neurons of Cln1 ko mice show highly upregulated synthesis of cholesterol without cholesterol accumulation (Ahtiainen et al., 2007), and increased plasma membrane expression of the α- and β-subunits of the F1 complex of the mitochondrial ATP syntase, suggested to function as an apoA-1 receptor (Lyly et al., 2008). Cultured Cln5 ko macrophages show increased cholesterol efflux as well as changes in sphingolipid trafficking (Schmiedt et al, 2012). In this study, possible changes in lipid metabolism were not assessed at the cellular level. However, plasma lipid changes seen in Cln1/5 dko mice resemble more the trends previously observed in Cln5 ko mice, whereas Cln1 ko mice seem to display the opposite changes involving decreased plasma cholesterol. (Lyly et al., 2008; this study).
Myelin membrane is mainly composed of lipids, and is especially enriched in cholesterol, galactolipids and glycosphingolipids (Chrast et al., 2011) and is therefore sensitive to disturbed lipid metabolism. NCL proteins have been suggested to play a role not only in lipid metabolism/transport but also in myelination processes (Schmiedt et al., 2012; Kuronen et al., 2012). Myelination was also shown to be affected in this study, since at the age of 3 month all three mutant mouse models showed fewer MBP positive fibers in the superficial laminae of S1BF compared to wt controls. Although lipid metabolism was not studied in the brain in this study, we postulate that the disturbances in lipid metabolism may affect the assembly of the myelin sheath, leading to defective myelination.

**Gene expression profiling of the Cln1/5 dko cortex implicates changes in genes involved in immune response, nerve ensheathment and transmission of nerve impulse**

Gene expression profiling of the Cln1/5 dko mouse cortex revealed changes in genes involved particularly in immune response, nerve ensheathment and transmission of nerve impulse. Consistent with our immunohistochemical analyses, which indicate defective myelination in the superficial cortical laminae, downregulation of key genes in myelin assembly (Opalin, Mal, Mag, Mog, Plp1, Mbp) was observed. These gene expression changes may be indicative of defects in oligodendrocyte development and function. Furthermore, recent studies have indicated that Cln5 has a specific role in oligodendrocyte maturation (Schmiedt et al., 2012), and it is possible that these NCLs share both neurodevelopmental and neurodegenerative aspects.

Interestingly, the most downregulated gene in the gene expression data was α-synuclein (Snca), which is upregulated in many neurodegenerative diseases, such as Parkinson disease (Vekrellis et al., 2011). Snca was also shown to be upregulated in Cln1 ko embryonic neuronal cell cultures.
(Ahtiainen et al., 2007). The level of soluble α-synuclein was also significantly reduced in another mouse model of NCL diseases, the cathepsin D ko mice, where the reduced neuropil staining was accompanied with aggregation of α-synuclein in inclusion bodies (Cullen et al., 2009). α-Synuclein is a presynaptic protein, which is an important regulator in dopaminergic transmission, synaptic plasticity, and vesicle trafficking (Uversky, 2008; Ruipérez et al., 2010). α-Synuclein deficient mice are viable, but they suffer from significant neurochemical, electrophysiological, and behavioral deficits (Abeliovich et al., 2000). Western blot analysis supports these microarray findings, showing almost complete lack of α-synuclein expression in the cerebral cortex of 1 and 3 month old Cln1/5 dko mice. Although immunohistological staining showed individual variability in the levels of α-synuclein protein in 1 and 3 month old Cln1/5 dko mice, these results support the Western blot findings, showing reduced α-synuclein staining in the cortex and slightly decreased expression in the hippocampus. We suggest that the individual variability may depend upon the stage of the disease, and the loss of α-synuclein expression in cortex could be linked to the early neuronal loss seen in this region in Cln1/5 dko mice. α-Synuclein may possibly have a role in NCL pathogenesis, or at least these disease mechanisms may have a significant impact upon α-synuclein expression. This protein also functions as a lipid-binding protein, and lipids can regulate its oligomerization into amyloid fibrils found in Lewy bodies (Chua et al., 2011).

Among the most upregulated genes in Cln1/5 dko were Cap1, complement C1q subcomponents Clqb and C1qc and GFAP. Cap1 has been implicated in a number of complex developmental and morphological processes, including endocytosis and actin polymerization (Sultana et al., 2005). Upregulation of Cap1 is evident in all three knock-out models (Cln1 ko, Cln5 ko and Cln1/5 dko), and it is clustered on the same chromosomal region as Ppt1 (gene mutated in Cln1
ko), indicating a possible functional relationship between these genes (Ahtiainen et al., 2007; von Schantz et al., 2008). C1q, a key player in classical complement pathway, is considered to be a marker for microglial activation (Färber et al., 2009), and is implicated in the pathogenesis of many neurodegenerative diseases (Nayak et al., 2010). Furthermore, it has been suggested that in the early stages of CNS neurodegenerative diseases, reactive astrocytes and C1q play a role in pathophysiological process that leads to synapse loss and neuronal death (Stevens et al., 2007). Indeed, the upregulation of GFAP expression is consistent with the marked astrocytosis seen in Cln1/5 dko brain. Our gene expression data provide further insight into the processes involved in the early stages of NCL pathogenesis. Upregulation of immune response pathways is consistent with the microglial activation we observed in the affected cerebral cortex. Decreased expression of myelin-specific genes provides evidence that defective myelination is due to a failure in myelin development processes. We suggest that NCL proteins may be linked to the development of the nervous system or involved in so far uncharacterized neuron-specific metabolic pathways.

**Conclusions**

Simultaneous deletion of two NCL genes, Cln1 and Cln5, has allowed us to show for the first time that early demyelination, cortical astrocytosis and microglial activation all occur prior to neuronal loss in this model. Together these results could be interpreted as further support for the existence of interactions between Cln1 and Cln5 gene products, since the loss of both Cln1 and Cln5 genes led to a more severe NCL phenotype in mice than the loss of only one of these genes. Alternatively, this could simply reflect that deficiency of these genes leads to convergent pathological pathways that have a more severe combined effect. Indeed, significant accumulation of autofluorescent storage material, the ultrastructure of storage material, increased astrocytosis
and microglial activation, as well as myelination defects seen in Cln1/5 dko mice resemble the more severe pathological features of Cln1 ko mice, whereas the onset of neuron loss is similar to that observed in Cln5 ko mice. Decreased α-synuclein gene and protein expression in the Cln1/5 dko brain is a novel finding that might reflect the early cortical neuron loss and needs further investigation. More detailed studies are needed to obtain a comprehensive view of NCL diseases and the neurodegenerative mechanisms involved in the development of these brain disorders.

METHODS

Generation of the Cln1/5 dko mouse model

Homozygous Cln1<sup>−/−</sup> (Cln1 ko) and Cln5<sup>−/−</sup> (Cln5 ko) mice (Mus musculus) were backcrossed to C57BL/6JRccHsd for more than 10 generations, and their congeneity was confirmed with the Mouse Medium Density SNP Panel (Illumina). Cln1 ko and Cln5 ko mice were crossed to initially generate heterozygous, and subsequently Cln1<sup>−/−</sup>/Cln5<sup>−/−</sup> double knock-out (Cln1/5 dko) offspring. These Cln1/5 dko offspring were viable and fertile. The genotypes of the mice were determined by PCR of DNA from tail biopsies. We used systematically sampled brain or skin tissue from E16.5-3 month old mice, with age- and sex-matched C57BL/6JRcc wild-type (wt) mice serving as a control strain. After sacrificing, body weight was determined. All animal procedures were performed according to protocols approved by the ethical boards for animal experimentation of the National Public Health Institute and University of Helsinki, as well as State Provincial offices of Finland (agreement numbers ESLH 2009-05074/STH 415 A and KEK10-059), and all experiments were performed in accordance with good practice of handling laboratory animals and genetically modified organisms.
**Generation and characterization of mouse iPS cells**

Mouse embryonic fibroblasts (MEFs) from both the *Cln1/5 dko* and wt mice (E16.5) were reprogrammed by using the retrovirus-mediated delivery of the four Yamanaka factors (Oct-3/4, Sox-2, Klf-4 and c-Myc, Addgene plasmids no. 13366, 13367, 13370 and 13375, described in Kitamura et al., 2003) according to protocol of Takahashi et al. (2007). iPS cell clones were grown on leukemia inhibitory factor (LIF)-producing SNL-feeder cells (McMahon and Bradley, 1990), kindly provided by the Wellcome Trust Sanger Institute (UK), collected on the basis of colony morphology (Fig. S1A), expanded and further purified by MACS anti-stage-specific embryonic antigen 1 (SSEA-1) MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Expression of SSEA-1 (Fig. S1A) and alkaline phosphatase (data not shown) in different clones was analyzed by ES cell characterization kit (Millipore, Billerica, MA). Expression of other ES cell marker genes (*Nanog, Rex-1, Fgf-4, Esg-1, E-ras*) in selected iPS cell clones was verified by RT-PCR (Fig. S1B) by using primer sequences earlier described by Takahashi et al. (2007).

**Embryoid body (EB) formation and analyses of EB size**

To minimize the inhibitory effect of the remaining feeder cells during EB formation, all iPS cell clones were purified by MACS anti-SSEA-1 MicroBeads (Miltenyi Biotech) before starting the experiments. The number of purified cells was analyzed by Nexcelom T4 cell counter (Nexcelom Bioscience, Lawrence, MA) and 1.5 x 10^6 cells/clone were plated on 60 mm Petri dish (Sterilin, Newport, UK) to facilitate EB growth in suspension in ES medium (DMEM+15% ES serum, 1x Glutamax, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol and antibiotics). Formation of EBs was followed daily by microscope. On day seven, EBs were transferred on 12-well plate for recording images with a Leica EC3 digital camera attached to
Leica DMIL microscope (Leica Microsystems, Wetzlar, Germany). The size of EBs was determined by the Las EZ programme (Leica Microsystems). The average size of EBs derived from different iPS cell clones was calculated from minimum of 40 different EBs. Statistical analyses of the EB sizes between Cln1/5 dko and wt clones were assessed using student's \( t \)-test for independent samples with unequal variances assumed. Statistical significance was considered at \( P < 0.05 \). \( P \)-values are two-sided and presented here as standard error of means (± SEM). EB formation was always done in pairs with Cln1/5 dko and wt clones. Results were verified in three independent experiments (by using three Cln1/5 dko and two wt clones).

**Differentiation of iPS cells into different germ layers**

To study the spontaneous differentiation potential of the produced iPS cell clones into the three primary germ layers, EBs were grown as described above for seven days and then plated on gelatin-coated tissue culture plates. Differentiation was followed daily by microscope. The cells were fixed with 4% paraformaldehyde (PFA) and stained with goat anti-FoxA2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-\( \beta \)-III tubulin (1:1000, Covance, Princeton, NJ) or rabbit anti-desmin (1:500, Epitomics, Burlingame, CA) antibodies for indication of endoderm, ectoderm and mesoderm, respectively (Fig. S2). Image processing (brightness and contrast) was performed with Adobe Photoshop and Adobe Illustrator (Adobe Systems Inc., San Jose, CA).

**Electron microscopy and autofluorescence of storage material**

For electron microscopy, the brains were removed from sacrificed 3 month old male Cln1 ko, Cln5 ko, Cln1/5 dko mice and wt controls. Bisected brains were immersion fixed in 0.1 M phosphate buffer pH 7.4/4% PFA/2.5% glutaraldehyde for two hours, incubated in fresh fix
solution for two days, and stored in 10 mM phosphate buffer at +4°C until processed further. 1-2 mm³ pieces were excised from corpus callosum, thalamus, brain stem and cerebral cortex. Pieces were osmicated, dehydrated and embedded in epoxy resin. After identification of the desired anatomical regions from thin sections stained with toluidine blue, ultra thin sections were cut and contrasted with uranyl acetate and lead citrate and viewed by JEOL 1400 Transmission Electron Microscope (JEOL Ltd, Tokyo, Japan), and images were taken with an Olympus-SIS Morada camera.

For autofluorescence analysis, Cln1 ko, Cln5 ko, Cln1/5 dko and wt mice were perfused with 4% PFA, and the brains were further immersion fixed in 4% PFA. Mouse brain sections spanning the S1BF and VPM/VPL were mounted onto a gelatin-chrome coated slides and coverslipped with Vectashield (Vector Laboratories, Peterborough, UK). Images from each section were captured at 63x magnification using a Leica SP5 confocal microscope and a 488 nm excitation laser (Leica Microsystems). Thresholding image analysis was performed to determine the extent of endogenous autofluorescence. Three animals per group were included in the analysis. Three consecutive sections spanning the S1BF or VPM/VPL were mounted onto a chrome-gelatin coated slide and coverslipped. The non-overlapping images from each section were captured at 40× magnification using a Leica SP5 confocal microscope and a 488 nm excitation laser. During image capture, all parameters and calibration were kept constant. Semi-quantitative thresholding image analysis was carried out using Image Pro Plus software (Media Cybernetics Chicago, IL) to determine the number of pixels per image that contained autofluorescent storage material.
**Histological processing and Nissl staining**

Brains from 3 month old Cln1 ko, Cln5 ko and Cln1/5 dko male mice and wt controls (n = 6 per genotype) were harvested as previously described (Schmiedt et al., 2012). Briefly, the mice were perfused with 4% PFA, and the brains were removed and postfixed in 4% PFA in 0.1 M sodium phosphate buffer (pH 7.4) overnight before cryoprotection in 30% sucrose in 50 mM Tris buffered saline (TBS) with 0.05% sodium azide, and then frozen in liquid nitrogen and stored at –80 °C. The brains were bisected along the midline and cut into 40 μm frozen coronal sections (Microm freezing microtome, Thermo Fisher Scientific, Waltham, MA). A series of every sixth section through each brain was mounted onto gelatin-chrome coated Superfrost microscope slides (VWR, Radnor, PA). Slides were air-dried overnight and incubated with 0.05% cresyl fast violet/ 0.05% acetic acid (VWR) in water for 45 min at 60°C, rinsed in distilled water and differentiated through an ascending series of alcohols, cleared in xylene, and coverslipped with DPX.

**Immunohistochemistry of frozen sections**

To investigate the extent of astrocytosis, microglial activation and myelination in 1 and 3 month old male Cln1 ko, Cln5 ko, and Cln1/5 dko and the wt control brains (n = 6 per genotype), a one in six series of free-floating frozen sections were immunohistochemically stained for glial fibrillary acidic protein (GFAP), cluster of differentiation (CD68) and myelin basic protein (MBP), respectively (as described previously in von Schantz et al., 2009 and Schmiedt et al., 2012). Briefly, sections were incubated in 1% hydrogen peroxide (H2O2) in TBS and blocked 40 min with TBS/0.3% Triton X-100 (TBST)/15% normal goat serum before incubation overnight in the following polyclonal primary serums: rabbit anti-GFAP (1:4000, DAKO, Glostrup,
Denmark), rat anti-CD68 (1:1000, Serotec, Oxford, UK), and rat anti-MBP (1:500, Millipore) diluted in TBS with 10% normal rabbit/rat serum and TBST. Sections were incubated for 2 h with secondary antiserum (biotinylated goat anti-rabbit IgG, 1:1000, Vector Laboratories) in TBS with 10% normal goat serum and TBST, and for 2 h in an avidin-biotin-peroxidase complex in TBS (Vectastain ABC Elite Kit, Vector Laboratories). Immunoreactivity was visualized by 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St. Louis, MO) and 0.001% H₂O₂ in TBS for 10 min. Sections were mounted on gelatin-chrome-coated Superfrost microscope slides (WVR), and coverslipped with DPX.

**Thresholding image analysis of astrocytic and microglial phenotypes**

The optical density of GFAP and CD68 immunoreactivity was assessed using semi-automated thresholding image analysis, as previously described (Bible et al., 2004; Pontikis et al., 2005). Three animals per group were included in the analysis. Briefly, 30 non-overlapping images, on triplicate sections, were taken through the thalamic VPM/VPL and cortical S1BF regions, starting from a defined anatomical landmark. Images were captured with Leica SP5 confocal microscope using a ×40 objective. Image Pro Plus image analysis software (Media Cybernetics) was used to determine the area of immunoreactivity by applying a threshold that discriminated staining from background in each image. Data were separately plotted graphically as the mean percentage area of immunoreactivity per field ± SEM for each region. Differences between groups were analyzed with one-way ANOVA (more than two groups) with post-hoc Bonferroni. The level of significance was set to $P < 0.05$. 
Immunohistochemistry of paraffin sections

α-Synuclein protein expression was analyzed from paraffin sections. Three month old male Cln1 ko, Cln5 ko, Cln1/5 dko and wt control mice (n = 6 per genotype) were included in the study. Brains were removed, bisected along the midline and immersion fixed with 4% PFA in PBS (pH 7.4) before embedding in paraffin. The tissue sections were cut into 5 µm sections and stained with α-synuclein antibody (rabbit polyclonal hSA-2, raised and affinity-purified at Open Biosystems, Inc., Huntsville, AL, http://www.openbiosystems.com) against recombinant, full-length α-synuclein. The antibody was a kind gift from prof. Michael G. Schlossmacher, University of Ottawa, Canada, and is described in Mollenhauer et al. (2008). Briefly, sections were deparaffinized, rehydrated in alcohol series, incubated in 5% H2O2 in methanol to quench endogenous peroxidase activity, and rinsed with TBS. After boiling in citrate buffer (pH 6.0) for 5 min, the sections were blocked with 10% goat serum in TBS and incubated overnight at +4°C with primary antibody (anti-mouse α-synuclein, 1:800) in 10% goat serum in TBS. Sections were washed with TBST (0.025%) and incubated with biotinylated secondary antibody (anti-rabbit IgG, 1:200 Jackson ImmunoResearch Laboratories, Inc, Reston, VA) in 10% goat serum in TBS for one hour. Sections were treated with avidin-biotin peroxidase mix (Vectastain ABC Elite Kit, 1:400, Vector Laboratories) for 40 min followed by washes with TBS. Antibody binding was detected with a standard DAB reaction (Sigma-Aldrich), after which the sections were counterstained for two min with hematoxylin (Sigma-Aldrich). After dehydration, the sections were cleared in xylene, air-dried and coverslipped with DPX.
Measurements of cortical thickness and regional volume

Stereological analysis was used to determine the thickness of the M1, S1BF, V1 and LEnt cortical layers, as well as cortical and hippocampal volumes. We used StereoInvestigator software (Microbrightfield, Williston, VT) to obtain unbiased Cavalieri estimates from 40 µm Nissl stained cryosections from Cln1 ko, Cln5 ko, Cln1/5 dko and wt male mice (n = 6 per genotype) at 3 months of age, with no prior knowledge of genotype. The boundaries of brain regions were defined by reference to landmarks described by Paxinos and Franklin (2001). All analyses were carried out on a Zeiss, Axioskop 2 MOT microscope (Carl Zeiss, Welwyn Garden City, UK) linked to a DAGE-MTI CCD-100 camera (DAGE-MTI, Michigan City, IN). Measurements of the thickness of M1, S1BF, V1 and LEnt were made in three consecutive Nissl stained sections with 10 measurements made from the white matter to the pial surface of the cortex within each section (Bible et al., 2004). Results were expressed as the mean cortical thickness (µm per region). Regional volumes of cortex and hippocampus were determined by placing an appropriately spaced sampling grid over sections and the number of points covering the relevant areas was assessed using a 2.5× objective. Results are expressed in µm³ and the mean volume of each region was calculated for each genotype. The mean coefficient of error (CE) for all individual Cavalieri estimates was calculated according to the method of Gundersen and Jensen (1987) and was ≤ 0.1 in all these analyses. Differences between groups were analyzed with one-way ANOVA with post-hoc Bonferroni (GraphPad Software Inc., La Jolla, CA). The level of significance was set to P < 0.05.
Counts of neuronal number

The design-based optical fractionator probe was used to estimate cell number in Nissl stained sections in cortical S1BF and VPM/VPL. Nissl stained cells were only counted if they had a neuronal morphology and a clearly identifiable nucleolus. A line was traced around the boundary of the region of interest, a grid was superimposed and cells were counted using a 100× objective within a series of dissector frames placed according to intersections of the sampling grid. Different grid and dissector sizes were determined according to each brain region using a CE value ≤ 0.1 to indicate sampling efficiency. Layer V of the S1BF grid was 175 × 175 µm, frame 70 × 40 µm, layer VI grid was 200 × 175 µm, frame 55 × 40 µm, and layer IV grid was 150 × 150 µm, frame 70 × 40 µm. For VPM/VPL the grid was 175 × 175 µm, frame 70 × 40 µm. A series of every sixth Nissl stained sections was used for all the brain regions. Differences between groups were analyzed with one-way ANOVA (more than two groups) with post-hoc Bonferroni. The level of significance was set to \( P < 0.05. \)

Plasma lipid analysis

1 month old female \( Cln5 \) ko, \( Cln1 \) ko and \( Cln1/5 \) dko mice \( (n = 8 \) per genotype) and wt controls were fasted for a 4-hour period and blood for plasma lipid and lipoprotein analysis was obtained. Iodoacetic acid (1 mM final) was immediately added to samples to avoid lecithin-cholesterol acyltransferase (LCAT) activity. Triglycerides and total cholesterol were measured using enzymatic methods (GPO-PAP 1488872 kit and CHOD-PAP 1489232 kit, respectively, Roche Diagnostics, Basel, Switzerland). Mouse apoA-1 was quantified by sandwich ELISA (van Haperen et al., 2000), and phospholipid transfer protein (PLTP) activity was measured using radiometric assay (Jauhiainen and Ehnholm, 2005). For the analysis of cholesterol distribution
in lipoproteins, plasma samples were pooled and fractionated by fast-performance liquid chromatography (FPLC) using high-resolution Superose 6 size-exclusion chromatography columns (GE Healthcare, Waukesha, WI) to separate the lipoprotein classes. Two pools per genotype (wt, Cln5 ko, Cln1 ko, and Cln1/5 dko) were analyzed (3 independent plasma samples per genotype). \( P \)-values were evaluated using one-way ANOVA, with \( P < 0.05 \) considered as statistically significant.

**Gene expression profiling**

Comparative gene expression profiling was performed from the 1 month old Cln1/5 dko male mice and wt controls (\( n = 6 \) per genotype). Cortices from the left hemisphere of wt controls and Cln1/5 dko mice were excised and the RNA was isolated with Qiagen RNA Lipid Tissue Kit (Qiagen, Hilden, Germany). Purity of RNA was verified with Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA) and its concentration was determined with NanoDrop (Thermo Fisher Scientific). 400 ng of total RNA was labeled and fragmented and hybridization, post-hybridization washes, staining and scanning were performed according to manufacturer’s instructions (Applied Biosystems Inc., Foster City, CA). Gene expression profiles were determined using the Illumina MouseWG-6 v2.0 Expression BeadChip and GenomeStudio Data Analysis Software (Illumina Inc., San Diego, CA). Gene expression analysis was performed with Chipster v1.4.7 analysis software (CSC, Espoo, Finland), and data normalization was carried out with quantile method. Quality control was performed to ensure the technical quality of the samples. \( P \)-values (adjusted with conditional testing) were used to determine the significantly up- and downregulated genes (\( P < 0.05 \)). Pathway analysis from the up- or downregulated genes (according to student’s \( t \)-test, \( P < 0.05 \)) was performed with DAVID Bioinformatics Resources.
6.7 software (Huang et al., 2009a, 2009b), and the Benjamini-Hochberg method was used for multiple hypothesis testing correction of the \( P \)-values.

**Quantitative real-time RT-PCR**

Quantitative real-time (RT)-PCR was performed as described previously (Schmiedt et al., 2012). Briefly, RNA from frozen cortices of 1 month old \( Cln1/5 \) dko \( (n = 6) \) and wt control \( (n = 6) \) mice was isolated as described in the previous paragraph. RNA was treated with DNase I (Qiagen) in order to eliminate genomic DNA. The reverse transcription reactions were carried out on 10 ng of RNA using TaqMan Reverse Transcription Reagents with random hexamer primers (Applied Biosystems, Foster City, CA). To average out the inter-individual variability, three different reverse transcription reactions were performed for each sample. TaqMan Gene Expression Array of selected gene was purchased from Applied Biosystems (Snca: Mm01188700_m1). The mRNA expression levels of this gene and a standard house-keeping gene, mouse TATA-box binding protein (Tbp: Mm00446970_m1), were quantified with ABI Prism 7000 sequence detection system (Applied Biosystems). The PCR reactions (10 µl) were carried out with TaqMan Universal Master Mix according to manufacturer’s instructions using the following parameters: 50º C for 2 min, 95ºC for 10 min, 40 cycles of 95ºC for 15 s, 60ºC for 1 min. Relative levels of the selected genes were calculated using the \( \Delta\Delta C_T \) method, as described previously (Kopra et al., 2004). The absolute change in expression level is given by \( 2^{-\Delta C_T} \).

**Western blotting**

Whole brains without cerebellum (denoted as “cerebrum”) and cortices of 1 and 3 month old \( Cln1 \) ko \( (n = 3) \), \( Cln5 \) ko \( (n = 3) \), \( Cln1/5 \) dko \( (n = 5) \) and wt control \( (n = 4) \) mice were
homogenized in extraction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.5% Triton X-100) supplemented with Protease Inhibitor Cocktail (Sigma-Aldrich) using Precellys CK14 lysing tubes (Bertin Technologies, France). The homogenates were clarified by centrifugation at 1000×g for 10 min in a Beckman TLA 100.3 rotor at +4°C. Total protein concentrations were estimated by DC Protein Assay (Bio-Rad, Hercules, CA) and equal amounts of total protein (30 µg) were analyzed by Western blotting. Protein samples were boiled and run on a 14% SDS-PAGE gel. After the transfer to nitrocellulose membrane (Hybond ECL, GE Healthcare), the blot was blocked in 5% milk powder in TBST and incubated with primary antibody (mouse-anti-α-synuclein, 1:1000, Cell Signaling, Danvers, MA) followed by the incubation with HRP-conjugated goat-anti-mouse secondary antiserum (1:5000, DAKO). Immunoreactivity was visualized by enhanced chemiluminescence assay (Western Lightning-ECL, PerkinElmer Inc., Waltham, MA). β-III tubulin (1:1000, Millipore) expression was used as a loading control. Densitometric analysis of the autoradiograms was performed by Image J 1.41o software. Student’s t-test was used for the statistical analyses. Three animals per group were included in the analysis.

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COMPETING INTERESTS

The authors declare no competing financial interests.

REFERENCES


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Clinical issue
Neuronal ceroid lipofuscinoses (NCLs), monogenic neurodegenerative diseases, offer a well-studied model to study the molecular events underlying neurodegeneration. The clinical phenotypes of NCLs are characterized by progressive loss of vision, mental and physical decline, epileptic seizures and premature death. The similar phenotypes of NCL mouse models, and the potential for interactions between NCL proteins, suggest that shared or convergent disease mechanisms may be involved. Analysis of disease mechanisms behind NCL will help to identify biomarkers that are important in monitoring the efficiency of different therapeutic strategies. Several promising therapeutic approaches are being investigated for the treatment of the NCLs, specifically for those caused by deficiency of soluble lysosomal proteins – like CLN1 and CLN5, investigated here. However, no curative therapies are currently available.

Results
Here, we developed a novel mouse model lacking both Cln1 and Cln5 genes. The resulting Cln1/5 double knock-out mouse was used as a model for studying disease mechanisms in the NCLs. The consequences of combined deficiency of CLN1 and CLN5 are of specific interest since these proteins may interact and common disease mechanisms have been suggested. The simultaneous loss of both Cln1 and Cln5 genes led to a more severe NCL phenotype in mice than the loss of only one of these genes. Cortical demyelination and more pronounced glial activation in cortical and thalamic regions was followed by cortical neuron loss. Specifically, microglial activation and lipid abnormalities were highlighted and the study also identified several dysregulated proteins with possible value as future biomarkers. Decreased α-synuclein gene and protein expression in the Cln1/5 double knock-out brain was a novel and interesting finding that needs further investigation.

Implications and future directions
This study demonstrates the use of the novel Cln1/5 double knock-out mouse model in identifying disease-modifying factors for NCL and other neurodegenerative diseases. Further studies using this double knock-out mouse model will help to more accurately identify the molecular defects underlying the CLN1 and/or CLN5 disease and to identify disease biomarkers. Deeper understanding of the biochemical and molecular cascade of events relevant to the pathogenesis of these diseases will be required to achieve significant therapeutic outcomes.
Figure legends

**Fig. 1** Impaired EB formation and differentiation of *Cln1/5* dko iPS cells. (A) Microscopic images reveal the smaller size and more irregular shape of *Cln1/5* dko EBs compared to wt EBs on day seven. (B) Statistical analysis of EB diameters shows significant difference in the EB size between wt and *Cln1/5* dko clones (student’s *t*-test). Columns present average (*n* ≥ 40) EB size, error bars represent SEM. *P* < 0.05, ***P* < 0.001. (C) Seven day old EBs were plated on gelatin-coated plates for spontaneous differentiation. Images show slower cellular outgrowth from *Cln1/5* dko EBs compared to wt EBs, indicating delayed spontaneous differentiation potential of *Cln1/5* dko EBs. Images were taken 3 days after plating. Scale bar 500 µm.

**Fig. 2** Accumulation of autofluorescent storage material in cerebral cortex and thalamus. (A) Confocal images of tissue sections from the cortex and thalamus show markedly increased autofluorescence in 3 month old *Cln1/5* dko mice. Scale bar 100 µm. (B) Thresholding image analysis revealed higher levels, although not significantly, of autofluorescent storage material in the cortex and thalamus of *Cln1/5* dko mice. (C) Electron micrographs of the storage material in the *Cln1/5* dko, *Cln1* ko and *Cln5* ko brains. (C.1) A cortical neuron of 3 month old *Cln1/5* dko mouse shows numerous storage bodies (s, indicated by arrows) and mitochondria (m) in the perinuclear area (N for nucleus). (C.2) At higher magnification, ultrastructure of the neuronal storage material is clearly visible, showing typical granular osmiophilic deposits, GRODs, with tiny grains inside. The storage material is surrounded by a double membrane. (C.3) Within the thalamus of 3 month old *Cln1/5* dko mouse, some neurons were loaded with storage bodies, which were more electron dense than those in the cerebral cortex. (C.4) Higher magnification of
the thalamic storage bodies shows typical GRODs surrounded by a double membrane. (C.5) Ultrastructure of the storage material within cortical neurons of 3 month old Cln1 ko mice closely resembles the GRODs seen in the cerebral cortex of Cln1/5 dko mice, although the storage deposits are less abundant in Cln1 ko mice compared to Cln1/5 dko mice at this age. (C.6) In the cortex of 3 month old Cln5 ko mice, the neuronal storage material is composed of membranous profiles with rectilinear and fingerprint-like features. Magnification x 10 000 in (C.1) and (C.3), x 40 000 in (C.2), (C.4), (C.5) and (C.6). Scale bars in (C.1) and (C.3) 2 µm; in (C.2), (C.4), (C.5) and (C.6) 1 µm.

**Fig. 3** Increased astrocytosis and widespread microglial activation in the thalamocortical system of 3 month old Cln1/5 dko mice. (A) Immunohistochemical staining for the astrocytic marker GFAP in 3 month old wt, Cln5 ko, Cln1 ko, Cln1/5 dko mice. Representative images from VPM/VPL and S1BF show pronounced and widespread astrocytosis in Cln1 ko and Cln1/5 dko brains, and elevated GFAP expression in the Cln5 ko brain. Insets from Cln5 ko, Cln1 ko and Cln1/5 dko show the morphology of GFAP positive hypertrophic astrocytes with enlarged soma and thickened processes. (B) Immunohistochemical staining for the microglial marker CD68 in 3 month old wt, Cln5 ko, Cln1 ko, Cln1/5 dko mice. Widespread activation of microglia in VPM/VPL and S1BF was evident in all three mouse models, being most elevated in Cln1 ko and Cln1/5 dko brain. Insets from Cln5 ko, Cln1 ko and Cln1/5 dko show the morphology of activated CD68 positive microglia, with swollen soma and shortened processes. Inset scale bars 50 µm. (C) Quantification of increased microglial activation (CD68 immunoreactivity) and (D) astrocytosis (GFAP immunoreactivity) in 3 month old Cln1/5 dko mice. Thresholding image analysis confirms the significantly increased GFAP expression in the thalamic VPM/VPL and
cortical S1BF regions of *Cln1/5* dko mouse brain, compared to wt controls. CD68 expression was significantly increased in the S1BF of *Cln1/5* dko mouse brain compared to wt brain. Error bars represent SEM, *P** <0.01, *P*** < 0.001.

**Fig. 4** Defective myelination in the cortical laminae of *Cln5* ko, *Cln1* ko and *Cln1/5* dko brain. (A) Immunohistochemical staining with MBP revealed loss of MBP immunopositivity in *Cln1/5* dko cortex at the age of 1 month, especially in the most superficial laminae of S1BF (laminae II and III). (B) All mouse models showed less MBP immunopositivity in the superficial laminae of S1BF than wt mice at the age of 3 months. Scale bar 100 µm.

**Fig. 5** Early loss of cortical neurons in *Cln1/5* dko mice. Unbiased optical fractionator estimates of the number of Nissl stained neurons of 3 month old mice in S1BF lamina IV (A), lamina V (B), lamina VI (C) and VPM/VPL (D) revealed significant loss of neurons in S1BF lamina VI of *Cln1/5* dko brain. No significant loss of cortical S1BF neurons in laminae IV and V, or thalamic VPL/VPL neurons, was observed. *P < 0.05.

**Fig. 6** Lipid profile analysis from 1 month old wt, *Cln1* ko, *Cln5* ko and *Cln1/5* dko mouse plasma. Cholesterol (Chol), phospholipid (PL), triglyceride (Trig) and apoA-1 levels (A) and PLTP activity (B) were determined from individual wt, *Cln1* ko, *Cln5* ko and *Cln1/5* dko mouse plasma samples. PL levels, as well as PLTP activity, were significantly increased in *Cln1/5* dko mice. Values plotted as mean SEM. *P* < 0.05, *P** <0.01, *P*** < 0.001.
**Fig. 7** Western blot analysis of α-synuclein levels in 3 month old wt and *Cln1*/5 dko mouse brains. (A) In the cortex, α-synuclein protein expression was completely absent in *Cln1*/5 dko brains compared to wt brains. (B) α-Synuclein expression in cerebral lysates was absent in three out of five *Cln1*/5 dko samples. (C) Quantification of α-synuclein protein expression. Densitometric analysis shows a significant decrease in the expression of α-synuclein in the *Cln1*/5 dko cortex and cerebrum compared to the wt brains. (D) Immunohistochemical analysis of α-synuclein in 3 month old wt, *Cln1* ko, *Cln5* ko and *Cln1*/5 dko brain. There was a marked reduction of α-synuclein staining in the neuropil of *Cln1*/5 dko cerebral cortex. Hippocampal α-synuclein staining was also decreased in the *Cln1*/5 dko brain. In addition, slightly reduced α-synuclein staining was observed in the cortical neuropil and hippocampus of the *Cln5* ko brain. Scale bar 50 µm. *P* < 0.05, **P** < 0.001.
Fig. 1

A

Disease Models & Mechanisms

B

DMM

Accepted manuscript

C

Disease Models & Mechanisms • DMM • Accepted manuscript

wt

Cln1/5 dko

cl.15

cl.19-2

cl.23

cl.20

μm

180

150

120

90

60

30

0

Clute 15

23

19-2

20

* * *

*
Fig. 2

Panel A shows images of the cortex and thalamus from different genotypes: wt, Cln5 ko, Cln1 ko, and Cln1/5 dko. The images depict changes in the tissue structure.

Panel B presents bar graphs showing the mean fluorescent units for cortex and thalamus across the same genotypes. The graphs indicate significant differences in fluorescence levels.

Panel C includes higher magnification images of the same genotypes, highlighting specific cellular structures and highlighting the differences observed in the tissue morphology.
Fig. 3

A

VPM/VPL

wt

Cln5 ko

Cln1 ko

Cln1/5 dko

S1BF

wt

Cln5 ko

Cln1 ko

Cln1/5 dko

B

VPM/VPL

wt

Cln5 ko

Cln1 ko

Cln1/5 dko

S1BF

wt

Cln5 ko

Cln1 ko

Cln1/5 dko
C

GFAP

Mean Immunoreactivity (%)

VPM/VPL

wt  Cln5 ko  Cln1 ko  Cln1/5 dko

S1BF

Mean Immunoreactivity (%)

wt  Cln5 ko  Cln1 ko  Cln1/5 dko

D

CD68

Mean Immunoreactivity (%)

VPM/VPL

wt  Cln5 ko  Cln1 ko  Cln1/5 dko

S1BF

Mean Immunoreactivity (%)

wt  Cln5 ko  Cln1 ko  Cln1/5 dko
Fig. 4

A

1 mo

Cln1/5 dko

wt

B

3 mo

Cln5 ko

wt

Cln1 ko

Cln1/5 dko
Fig. 5

A

S1BF lam IV

B

S1BF lam V

C

S1BF lam VI

D

VPM/VPL

* Estimated neuron number
**Fig. 7**

**A**

3 mo ctx

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α-synuclein  βIII-tubulin

**B**

3 mo cerebrum

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α-synuclein  βIII-tubulin

**C**

![Graph showing data for 3 mo ctx and 3 mo cerebrum](graph)

- **wt**
- **Cln 1/5 dko**

**D**

![Images of tissue sections for Ctx and Hippocampus](images)
Table 1 Up- and downregulated genes in 1 month old Cln1/5 dko cortex

**Upregulated genes**

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FC, fold change; Adj. P-value, adjusted P-value
### Downregulated genes

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FC, fold change; Adj. P-value, adjusted P-value
Table 2 Up- and downregulated pathways in 1 month old Cln1/5 dko cortex

*Upregulated pathways*

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FC, fold change

*Downregulated pathways*

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<td>1.50E-05</td>
<td>-12.3</td>
</tr>
</tbody>
</table>

FC, fold change
Table 3 Overview of neurological and neuropathological changes in *Cln1* ko, *Cln5* ko and *Cln1/5 dko* mouse models

<table>
<thead>
<tr>
<th></th>
<th><em>Cln1 ko</em></th>
<th><em>Cln5 ko</em></th>
<th><em>Cln1/5 dko</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Onset of symptoms</strong></td>
<td>5 months</td>
<td>8 months</td>
<td>3 months</td>
</tr>
<tr>
<td><strong>Phenotype</strong></td>
<td>Motor abnormalities, seizures&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Motor abnormalities, seizures&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Motor abnormalities, seizures&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ultrastructure of storage material</strong></td>
<td>GROD&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Rectilinear, curvilinear, fingerprint&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GROD&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Onset of neuron loss</strong></td>
<td>Thalamus (4 months)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Cortex lam IV and V (4 months)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Cortex lam VI (3 months)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Onset of glial activation</strong></td>
<td>Cortex and thalamus (3 months)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cortex and thalamus (3 months)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cortex and thalamus (1 months)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Onset of myelination defects</strong></td>
<td>Cortex (3 months)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Cortex (1 month)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Cortex (1 month)&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Jalanko et al., 2005 and unpublished; <sup>b</sup>Kopra et al., 2004 and unpublished; <sup>c</sup>von Schantz et al., 2009; <sup>d</sup>Schmiedt et al. 2012; <sup>e</sup>this study
**Fig. S1.** Cln1/5 dko and wt mouse iPS cell clones show ES cell like morphology and express markers characteristic for ES cells. MEFs from both the wt and Cln1/5 dko mice were reprogrammed by using the retrovirus-mediated delivery of the four Yamanaka factors (Oct3/4, Sox2, Klf4 and c-Myc). (A) iPS cell colonies were collected on the basis of ES cell morphology (round shape and sharp borders) and the expression of SSEA-1 surface protein (red). (B) Expression of ES cell specific markers Nanog, Rex-1, Fgf-4, Esg-1 and E-Ras was detected in all clones by RT-PCR (see Materials and Methods for details). ES cells served as a positive control and MEFs as a negative control.
Fig. S2. Spontaneous differentiation of mouse induced pluripotent stem (iPS) cell clones. iPS cell clones (wt and Cln1/5 dko) were differentiated via EB formation (see Materials and methods). Seven day old Cln1/5 dko and wt EBs were plated on gelatin-coated plates and allowed to undergo spontaneous cell differentiation. The cells were fixed with 4% PFA, and differentiation into cell derivatives of all three germ layers was confirmed by staining with antibodies for FoxA2 (staining nucleus, endoderm), β-III tubulin (staining neuron-specific microtubules, ectoderm) or desmin (staining cytoplasm, mesoderm). Scale bar: 50 mm.
**Fig. S3.** Significant loss of body weight in *Cln1* ko, *Cln5* ko and *Cln1/5* dko mice compared to wt mice at 3 months of age. *P*<0.05, **P*<0.01.

**Fig. S4.** Immunohistochemical staining with CD68 reveals increased microglial activation in the *Cln1/5* dko cortex (S1BF) and thalamus (VPM/VPL) that was already detectable at 1 month of age. Compared to the wt, *Cln1/5* dko microglia displayed more intense CD68 immunoreactivity with enlarged soma and protracted processes in the S1BF and VPM/VPL. Higher magnification insets show the different morphologies of wt and *Cln1/5* dko microglia. Inset scale bar: 50 mm.
Fig. S5. Measurements of cortical thickness and regional volumes in 3 month old Cln1/5 dko brain. (A) Laminar thickness measurements in Cln1 ko, Cln5 ko, Cln1/5 dko and wt controls in cortical regions M1, S1BF, V1 and LEnt revealed no significant cortical thinning in Cln1/5 dko brain, whereas Cln5 ko displayed significantly increased thickness in S1BF, M1 and V1. (B) Unbiased Cavalieri estimates of regional volumes revealed no significant atrophy in the cortex or hippocampus of Cln1/5 dko mice. In Cln5 ko brain, the cortical volume was significantly increased. *P<0.05, **P<0.01, ***P<0.001.
Fig. S6. Distribution of cholesterol, phospholipids (PL), apoA-1 and triglycerides in HDL, VLDL or pre-β-HDL particles. Pooled plasma samples from wt and Cln1/5 dko mice were fractionated with FPLC. (A) Cholesterol and phospholipid distribution, as well as apoA-1 (B) and triglyceride (C) distribution in HDL, VLDL or pre-β-HDL was measured. In Cln1/5 dko plasma, cholesterol levels were elevated in HDL, and slightly decreased in VLDL particles. ApoA-1 levels were increased in HDL particles, whereas triglyceride levels were elevated in VLDL particles.
Fig. S7. (A) Expression of Snca in the wt and Cln1/5 dko cortex. Snca mRNA was quantified in the cortex of 1 month old mice using RT-PCR. Mean ± s.e.m., P-value was evaluated using Student’s t-test. Expression levels are expressed as the relative copy number compared with the Tbp transcript using the comparative threshold cycle (CT) method. (B) Western blot analysis from the cortical lysates shows a complete loss of α-synuclein protein in the 1 month old Cln1/5 dko mice compared to wt brains. (C) α-Synuclein expression in cerebral lysates was significantly reduced in 1 month old Cln1/5 dko samples. (D) Quantification of α-synuclein protein expression. Densitometric analysis shows a loss of α-synuclein expression in the 1 month old Cln1/5 dko cortex compared to the wt brains. Significant reduction of α-synuclein expression was also observed in the 3 month old Cln1/5 dko cerebrums. P<0.05, P***<0.001.
Fig. S8. Western blot analysis from the cerebral lysates shows no significant changes of α-synuclein protein in the 1 month (A) and 3 month (B) old Cln1/5 dko mice compared to wt brains.
Table S1 Expected Mendelian ratios and observed numbers of the *Cln1/5* dko offspring. In general, less double homozygous mice were born than expected.

<table>
<thead>
<tr>
<th>Breeding type</th>
<th>No. of offspring</th>
<th>Expected double homozygote offspring</th>
<th>Observed double homozygote offspring</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Cln1^{+/+}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>221</td>
<td>6.25%</td>
<td>7.24%</td>
<td>+ 0.99 %</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>71</td>
<td>25%</td>
<td>16.9%</td>
<td>- 8.1%</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>42</td>
<td>25%</td>
<td>14.3%</td>
<td>- 10.7%</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>34</td>
<td>25%</td>
<td>14.7%</td>
<td>- 10.3%</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{'-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>117</td>
<td>25%</td>
<td>22.2%</td>
<td>- 2.8%</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>16</td>
<td>50%</td>
<td>0%</td>
<td>- 50.0%</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>5</td>
<td>50%</td>
<td>40%</td>
<td>- 10.0%</td>
</tr>
<tr>
<td>$Cln1^{+/-}/Cln5^{+/-}$ x $Cln1^{+/-}/Cln5^{+/-}$</td>
<td>50</td>
<td>100%</td>
<td>88.5%</td>
<td>- 11.5%</td>
</tr>
</tbody>
</table>

*a*total number of born offspring; *b*expected number of offspring according to Mendelian ratio; *c*observed number of offspring; *d*percentual difference between the number of expected and observed double homozygote offspring.