Behavioral deficits, early gliosis, dysmyelination and synaptic dysfunction in a mouse model of mucolipidosis IV

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Abstract

Mucolipidosis IV (MLIV) is caused by mutations in the gene MCOLN1. Patients with MLIV have severe neurologic deficits and very little is known about the brain pathology in this lysosomal disease. Using an accurate mouse model of mucolipidosis IV, we observed early behavioral deficits which were accompanied by activation of microglia and astrocytes. The glial activation that persisted during the course of disease was not accompanied by neuronal loss even at the late stage. In vivo [Ca2+]imaging revealed no changes in resting [Ca2+] levels in Mcoln1−/− cortical neurons, implying their physiological health. Despite the absence of neuron loss, we observed alterations in synaptic plasticity, as indicated by elevated paired-pulse facilitation and enhanced long-term potentiation. Myelination deficits and severely dysmorphic corpus callosum were present early and resembled white matter pathology in mucolipidosis IV patients. These results indicate the early involvement of glia, and challenge the traditional view of mucolipidosis IV as an overtly neurodegenerative condition.

Keywords: Mucolipidosis IV, Lysosomal storage disease, Neuropathology, In vivo Ca2+ imaging, Glia

Introduction

Mucolipidosis IV (MLIV) is a lysosomal storage disorder (LSD) with autosomal recessive inheritance caused by loss of function of mucolipin-1 (also known as TRPML1). Over 20 different MCOLN1 gene mutations have been identified in MLIV patients, although the two founder mutations, both leading to complete loss of mRNA and functional protein, account for ~95% of all MLIV alleles and show a carrier frequency of 1:100 in the Ashkenazi Jewish population [1]. The most profound clinical manifestations of MLIV are severe psychomotor retardation during the first year of life and slowly progressing vision loss due to corneal clouding and retinal degeneration resulting in blindness by the second decade of life [2-4]. Neuromotor abnormalities include delayed attainment of motor milestones, spasticity, hypotonia, inability to walk independently, ptosis, myopathic facies, drooling, difficulties in chewing and swallowing, and severely impaired fine-motor function. Most patients reach a level of motor, speech and cognitive development of about 15 months, and remain neurologically stable during second and third decade of life [5,6]. The most striking MRI findings in MLIV include a dysplastic corpus callosum, widespread white matter abnormalities, including abnormal diffusion weighed imaging values: increased mean diffusivity and decreased fractional anisotropy, decreased T2 signal intensities in the thalamus due to increased ferritin deposition, and cerebellar atrophy in older patients [6-8]. At the cellular level MLIV results in the formation of abundant electron-dense inclusions composed of lipid-like (lamellated) and polysaccharide-like (granular) material, referred to as “compound bodies” [9,10].

TRPML1 functions as a non-specific cation channel [11-13]. Previous studies by our group and others showed TRPML1 localization to late endosomes and lysosomes (LEL) [14-17], and its involvement in lipid trafficking [18,19]; Ca2+-dependent LEL fission-fusion events [19]: formation of lysosomes from endosome-lysosome hybrids...
[17,20] and autolysosomes [21,22]; and lysosomal exocytosis [23,24]. However, the endogenous localization and function of TRPML1 in both neurons and glial cells in the brain have yet to be defined.

A mouse model of MLIV created in our laboratory was shown to accurately recapitulate the key features of the human disease [25]. At birth, the Mcoln1 knock-out mice display no overt phenotypes; limb weakening is observed at the age of 3 months and progresses to total hind limb paralysis and death by approximately 8 months. Ultrastructural analysis revealed presence of the storage inclusions in neurons and all types of glial cells. Inclusions resemble the “compound bodies” reported in MLIV patients and are detectable in the embryonic mouse brain. Histological analysis of end-stage brains revealed accumulation of gangliosides, cholesterol and P62/SQSTM1, as well as glial activation and reduced myelination [26]. The mechanisms that link loss of TRPML1 with brain pathology and the devastating neurological symptoms of MLIV remain unclear, and there is currently no treatment for the disease. Therefore, study of the MLIV mouse model is an essential step toward understanding disease pathogenesis and for testing potential therapies.

Here we investigate the onset and progression of neuronal and glial pathological mechanisms that link loss of TRPML1 with brain pathology and the devastating neurological symptoms of MLIV. Our data indicate an early and profound involvement of glial cells in pathogenesis of MLIV, a disease traditionally viewed as being “neuronal”, and provide new clues to the development of therapies for this devastating disorder.

Materials and methods

Animals

Mcoln1 knock-out mice were maintained and genotyped as previously described [25]. The Mcoln1<sup>−/−</sup> breeders for this study were obtained by backcrossing onto a C57Bl6N background for more than 10 generations. Mcoln1<sup>/+</sup> littermates were used as controls. Experiments were performed according to the institutional and US National Institute of Health guidelines and approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Open field testing

Open field testing was performed on naive male mice at one (n = 13, wild type; n = 9, Mcoln1<sup>−/−</sup>) and two (n = 17, wild type; n = 18, Mcoln1<sup>−/−</sup>) months of age under regular light conditions. Each mouse was placed in the center of a 27 × 27 cm<sup>2</sup> Plexiglas arena, and the horizontal and vertical activity were recorded by the Activity Monitor program (Med Associates). Data were analyzed during the first 10 mins in the arena. Zone analysis was performed to measure movements/time spent in the central (8 × 8 cm<sup>2</sup>) versus peripheral (residual) zone of the arena. Data were analyzed by two-way ANOVA (genotype × age) followed by Bonferroni post-test.

Stereological analysis and immunohistochemistry

To obtain brain tissue for histological examination two (n = 6 per genotype), three (n = 3, WT; n = 4, Mcoln1<sup>−/−</sup>) and seven month-old Mcoln1<sup>−/−</sup> and control mice (n = 4 per genotype) were transcardially perfused under isoflurane anesthesia with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were postfixed in 4% paraformaldehyde in PBS for 24 hours, washed with PBS, cryoprotected in 30% sucrose in PBS overnight, frozen in isopentane and stored at −80°C. Brains were bisected along the midline and one hemisphere was examined histologically. 40 μm coronal sections were cut using a Microm freezing microtome and collected into 96 well plates containing TBSAF (TBS, 30% ethylene glycol, 15% sucrose, 0.05% sodium azide). These sections were stored at 4°C prior to any staining procedures. For Nissl staining a one-in-six series of sections were mounted onto chrome gelatine coated slides, incubated in 0.05% cresyl violet stain and 0.05% acetic acid at 60°C for 30 mins before being dehydrated in ascending concentration of industrial methylated spirits (IMS) followed by xylene. The sections were then coverslipped using DPX mountant.

Quantitative histological measurements

Nissl stained sections were used to obtain cortical thickness measurements using Stereoinvestigator software. All analyses were carried out using ×2.5 objective on a Zeiss, Axiostop2 MOT microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). Briefly, within each region three consecutive sections were chosen and 10 perpendicular lines were drawn from the pial surface to the white matter.

Unbiased Cavalieri estimates of regional volume were obtained using Stereoinvestigator software. Briefly, for each of the regions a sampling grid size of 150 μm was superimposed on every one-in-twelfth Nissl stained section (cortex and hippocampus) or every one-in-six Nissl stained section (thalamus and striatum) and the number of points that fell within this region was recorded to provide an unbiased Cavalieri estimate of regional volume (μm<sup>3</sup>) for each brain. All measurements were carried out at ×2.5 objective on the Olympus BX50 microscope (Olympus Microscopes, South-on-Sea, UK).

Unbiased optical fractionator estimates for the number of Nissl stained neurons within the VPL-VPm, laminae IV and V of the S1BF, DLG and red nucleus were obtained using the Stereoinvestigator program. A grid size of 175 μm × 175 μm was used for the thalamus, 225 μm
\( \times 225 \, \mu m \) was used for laminae IV and V of the S1BF, and 125 \( \mu m \times 125 \, \mu m \) was used for the DLG and red nucleus. A counting frame of 68 cm \( \times \) 38 cm was used for all regions and all measurements were carried out at \( \times 100 \) objective on a Zeiss, Axioskop2 MOT microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). The mean coefficient of error (CE) for optical fractionator estimates of neuron number were all between 0.05 and 0.1. All analyses were performed blind to genotype, which was only revealed once these analyses were complete. At this point the mean value for each parameter was calculated for both Mcoln1-deficient and \(+/+\) mice. Differences between genotypes were compared statistically using Student’s \( t \)-tests with a P-value of \( \leq 0.05 \) considered as statistically significant.

**Immunostaining**

Immunostaining was performed as described [27]. The primary polyclonal rat anti-CD68 (Serotec, 1:2000) and polyclonal rabbit anti-GFAP (DAKO, 1:4000) antibodies were used. For myelin FluoroMyelin™ Green fluorescent stain (Molecular Probes) was used according to the manufacturer protocol.

**Thresholding image analysis**

Three consecutive GFAP- and CD68- immunostained sections were chosen for each of the regions being observed and 30 non-overlapping images were captured at 40\( \times \) objective by a live video camera (JVC, 3CCD, KY-F55B) mounted on Zeiss Axioplan microscope (West Germany). The lamp intensity, video camera setup, and calibration were kept constant when capturing all the images. These images were then analysed on ImageProPlus software with an appropriate threshold selected to distinguish the foreground immunostaining above the background for each of the age groups being observed. The data obtained from the thresholding analysis was plotted graphically as a mean percentage area of immunoreactivity per image.

**In vivo imaging of \( \text{Ca}^{2+} \) concentrations in cortical neurons**

To deliver the genetically encoded calcium indicator, craniotomies were performed on \( \text{Mcoln1}^{-/-} \) (n = 5) and control mice (n = 3) of 2 months and 1 week of age. A 5-mm diameter skull flap centered over the right primary somatosensory cortex was removed and 2 \( \mu l \) of AAV2/8 viral vector (5.6 \( \times \) 10^{12} viral genomes/ml) encoding YC3.60 under hybrid cytomegalovirus (CMV) immediate-early enhancer/chicken \( \beta \)-actin promoter/exon1/intron [28] were injected into the open brain close to the center of the craniotomy at a depth of about 0.5 mm and at a speed of 0.2 \( \mu l/min \). The brain surface (still covered by intact dura matter) was kept moist with Ringer’s solution at all times. Following the virus injection, the craniotomy was sealed with a glass coverslip and cemented with dental acrylic. Appropriate anesthetic and analgesic regimes were followed before, during and after the surgery. 3 weeks after the surgery, when the mice reached 3 months of age, they were anesthetized (isoflurane in balanced oxygen: 4\% for 5 min induction, then maintained at 1.2\%); head fixed and imaged on a multiphoton microscope. Our imaging setup was described earlier [29]. The excitation laser was tuned to 860 nm and the output power before the objective was set to 30 mW. Emitted light was collected in three channels: 460 – 500 nm (cyan channel (C), CFP fluorescence), 530 – 560 nm (yellow channel (Y), YFP fluorescence) and 575 – 630 nm (red channel (R), autofluorescence from storage material). With these settings, autofluorescent storage material yielded equal signals in cyan, yellow and red channels. Z-stacks were acquired with the resolution of 0.5 \( \mu m \)/voxel in X-Y dimension and the Z-step of 3 \( \mu m \) for imaging of neuronal cell bodies in the cortical layer II/III (512 \( \times \) 512 \( \times \) 270 \( \mu m \) stack) and with the resolution of 0.25 \( \times \) 0.25 \( \times \) 2 \( \mu m \)/voxel for imaging of dendrites in the cortical layer I (512 \( \times \) 512 \( \times \) 60 \( \mu m \) stack). Imaging settings were kept constant across mice.

Image processing was performed using the Fiji package of NIH ImageJ software (fiji.sc; rsbweb.nih.gov/ij) and MATLAB (MathWorks). Ratio of YFP to CFP signals representative of intracellular calcium concentration was calculated after subtracting the signal from background-subtracted red channel: \( R = \frac{X}{R} \). Regions of interest (ROIs) outlining cell bodies and dendrites were selected on the raw images and applied to the ratio images and to the red channel to estimate the amount of autofluorescent storage. The values for individual cells and dendrites were calculated as mean ratio in ROIs. All statistical tests were performed on \( R \) values. To convert \( R \) values to \([\text{Ca}^{2+}]\) for data presentation and interpretation, the following formula was used: \([\text{Ca}^{2+}] = K_d \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right)^{1/n} \). Values for \( K_d \) (277 nM) and the Hill coefficient \( n \) (1.1) were described previously [28]. \( R_{\text{min}} \) (0.67) and \( R_{\text{max}} \) (2.55) were measured experimentally. To create pseudocolored images, ratio images coded with “Rainbow RGB” lookup table were converted to the RGB space weighted by intensity.

The normality of datasets of YC3.60 ratio values was tested using Kolmogorov-Smirnov method. Since the distributions were found to be non-normal, they were compared between genotypes using the Wilcoxon rank-sum test with correction for clustering of values within individual mice [30]. The correlation of YC3.60 ratio values with the amount of cytosolic storage material was performed using a mixed effects model, with random slope effects for mouse, and using mouse-specific standardized
transformations of YC3.60 ratio and autofluorescence values.

Electrophysiology
Transverse hippocampal slices were prepared from 7 month-old *Mcoln1*−/− and control male mice (n = 6 per genotype) and field recordings were performed as described [31]. Data were normalized to the baseline response and are presented as group means ± SE. One-way ANOVA and Student’s t-test were used to determine statistically significant differences. For all experiments the experimenter was blind to genotype.

Electron microscopy
Seven month-old *Mcoln1*−/− and control mice (n = 4 per genotype) were anesthetized with isoflurane and transcardially perfused with PBS followed by 2% paraformaldehyde/2% glutaraldehyde in PBS, brains were removed and 50 μm coronal serial vibratome sections were processed for EM as previously described [32]. 35 micrographs per sample from stratum radiatum per mouse were obtained on a JEOL JEM-1011 transmission electron microscope at final magnification of ×25,000. Only asymmetric macular (non-perforated) synapses that were fully present on the micrograph were included in the analysis. Digital image analysis was performed using ImageJ (NIH). The post-synaptic density length was measured. Analysis of mitochondrion volume was performed as described [33]. For myelin sheath thickness measurements two perpendicular lines were drawn through the center of each myelinated axon and four measurements of the thickness were taken at each intersection with the myelin sheath with the mean value of four measurements calculated. The normality of all data sets was tested using the Kolmogorov-Smirnov test. Non-parametric data are presented as median values with interquartile ranges. An extended Wilcoxon rank-sum test that accommodates clustered data was used for comparisons between control and *Mcoln1*−/− groups for non-normally distributed data. Statistical significance threshold was set at p < 0.05.

Results

Early motor and cognitive deficits in *Mcoln1*−/− mice
To determine if *Mcoln1*−/− mice display early locomotor and behavioral deficits, spontaneous activity of one- and two-month-old naive *Mcoln1*−/− mice and wild-type littermates was tested in the open field arena. We found a significant decline in both jumping and vertical activity and an increase in the resting time in *Mcoln1*−/− mice at two months of age (Figure 1A-E), which indicates the early onset of motor deficits. Analysis of activity within the central zone revealed that the number of central zone entries, percentage of central track length and percentage of time spent in the center were significantly lower in *Mcoln1*−/− mice compared to wild-type controls at 2 months of age (Figure 1F-H) indicating an early decline in the exploratory activity associated with loss of *Mcoln1*.

Absence of brain atrophy in *Mcoln1*−/− brain
Neuropathology in models of lysosomal storage disorders is typically characterized by atrophy and neuronal loss within specific brain regions. To assess the progression of anatomical changes in the *Mcoln1*−/− brain, we performed unbiased stereological measurements of the volume of four brain regions (cortex, hippocampus, thalamus and striatum) in two, three and seven month-old mice. We observed no atrophy of any of these brain regions at any of these three time points (Additional file 1: Figure S1A). Next we assessed whether more subtle effects were evident within the cortex of these mice by obtaining cortical thickness measurements from the somatosensory barrelfield cortex (S1BF), primary motor cortex (M1), lateral entorhinal cortex (LEnt) and primary visual cortex (V1) at two, three and seven months. No significant changes in cortical thickness were detected between *Mcoln1*−/− and control mice at any age (Additional file 1: Figure S1B), suggesting that none of the examined regions of cortex undergoes overt neurodegeneration during the course of this disease.

Profound early activation of microglia and astrocytes in *Mcoln1*−/− mice
Since pronounced activation of microglia and astrocytes has been reported in the single available autopsy report from an MLIV patient [9], and in end-stage *Mcoln1*−/− mice [26], we evaluated the onset and progression of these phenotypes in brain tissue from two, three and seven month-old *Mcoln1*−/− mice. Immunostaining for the astrocyte marker GFAP revealed marked reactive astrogliosis in *Mcoln1*−/− brains as early as two months of age. At this time we observed an intense GFAP immunoreactivity in the somatosensory barrelfield cortex, primary motor cortex and hypothalamus, with the most prominent staining intensity in the VPL/VPM region of thalamus (Figure 2A, B). Astrocytosis in *Mcoln1*−/− mice progressed with age, and by the age of seven months intensely stained GFAP-positive cells were present throughout the entire area of thalamus and hypothalamus. These GFAP-positive astrocytes within the *Mcoln1*−/− brain appeared hypertrophied with enlarged soma and thickened processes. However, astrocytosis appeared to progress at different rates between brain regions. For example, staining for GFAP was more pronounced in VPL/VPM at earlier stages of disease, and only subsequently became more evident in S1BF, the target cortical region for this somatosensory relay nucleus of the thalamus (Figure 2C). Thresholding image analysis in the VPL/VPM area of thalamus...
revealed a significant effect of age and genotype on GFAP staining intensity ($F_{\text{genotype} \times \text{age}} = 5.67; p < 0.0107$) with increased staining in $Mcoln1^{-/-}$ mice at two and three months of age (post-hoc t-test with Bonferroni correction: $p_{2 \text{ months}} < 0.05$; $p_{3 \text{ months}} < 0.001$; $p_{7 \text{ months}} > 0.05$). GFAP staining intensity in S1BF cortex was also significantly affected by genotype and age ($F_{\text{genotype} \times \text{age}}=5.54; p = 0.0117$), however post hoc test revealed significant increase in GFAP in $Mcoln1^{-/-}$ mice only at the age of seven months ($p < 0.01$).

To examine the microglial response, brain tissue from two, three and seven month-old $Mcoln1^{-/-}$ mice and wild-type littermates was immunostained for the microglia/macrophage marker CD68. Compared to the wild-type controls, the $Mcoln1^{-/-}$ mice displayed pronounced activation of microglia early in disease progression, which was evident at two months of age, especially within the VPL/VPM region of thalamus followed by two-way ANOVA test showed a significant effect of genotype ($F_{\text{genotype}} = 11.54; p = 0.0027$), and no effect of age ($F_{\text{age}} = 0.098; p = 0.91$) on CD68 staining intensity (Figure 2F). Analysis of CD68 staining in the S1BF region of the cortex revealed significant effect of genotype and age ($F_{\text{genotype} \times \text{age}} = 4.1; p = 0.03$), with a significant increase in CD68 staining intensity at two ($p < 0.001$) and three ($p < 0.001$), but not at seven months (Figure 2E, F), when activated microglia appeared to be more localized to lamina IV. Similar changes in microglia morphology and increased staining intensity were observed with the microglial marker Iba1 (Additional file 2: Figure S2).

Ultrastructural analysis of the brain tissue (CA1 stratum radiatum of hippocampus) at seven months revealed accumulation of electron-dense storage bodies in $Mcoln1^{-/-}$ astrocytes similar to those described previously in the cortex [25] (Additional file 3: Figure S3A). We also observed the formation of large aggregates of electron-dense organelles (or clumps). Most often they were engulfed by the
Figure 2 (See legend on next page.)
plasma membrane which lacks synaptic contacts and, based on cell morphology [34], most likely belongs to microglia/macrophages (Additional file 3: Figure S3B).

**Normal resting calcium concentrations in the somatosensory cortex of Mcoln1−/− mice**

To assess if loss of TRPML1 and the early activation of microglia resulted in sub-lethal neurotoxicity and impaired calcium homeostasis, we performed *in vivo* measurements of intracellular calcium concentrations in cortical neurons of Mcoln1−/− and wild-type mice expressing the genetically-encoded ratiometric calcium sensor YC3.61 [28,35]. An adeno-associated viral vector (AAV 2/1) was used to deliver YC3.61 to layer II/III neurons of the somatosensory cortex, a region where pronounced astrogliosis and microglial activation is present (Figure 2). Resting [Ca2+]i was determined in the individual cell bodies and dendrites by *in vivo* multiphoton imaging of the injected area through implanted cranial window [28] in anesthetized mice. Analysis of resting calcium concentration in three month-old mice revealed no differences in either neuronal somata in layers II/III (Figure 3A, C) (3 WT mice, 888 cells; 5 KO mice 1875 cells analyzed) or neuropil in layer 1 (Figure 3B, D) (3 WT mice, 312 neurites; 5 KO mice 536 neurites analyzed). Resting [Ca2+]i levels in the dendrites of both KO mice and control littersmates were in good concordance with the previously reported values for wild-type mice [28]. Additionally, we observed large autofluorescent storage aggregates in the cytosol of Mcoln1−/− neurons (Figure 3E) which, however, had very little effect on the levels of intraneuronal resting [Ca2+]i (R = −0.074, p = 0.04), and even the cells with the highest amount of storage material had physiologically normal [Ca2+]i levels (Figure 3E, F). These data show that the loss of TRPML1 does not result in neuroexcitotoxicity and provides evidence of physiological health of Mcoln1−/− neurons at three months of age despite profound activation of microglia and astrocytes in the somatosensory cortex at this age.

**Activation of microglia and astrocytes is not accompanied by neuronal loss in Mcoln1−/− mice**

Activation of microglia has been linked to neuron loss in many neurodegenerative diseases [36]. To assess if chronic activation of microglia was associated with neuronal loss in Mcoln1−/− mice later in disease progression, unbiased optical fractionator neuron counts were obtained in the brain areas with the most prominent microgliosis, i.e. the VPL/VPM region of thalamus, laminae IV and V of the somatosensory barrelfield cortex, the CA1 and CA3 subfields of the hippocampus, the DLG and red nucleus, in seven month-old mice (Additional file 4: Figure S4). We observed no significant differences in neuron number between wild type and Mcoln1−/− mice in any of the examined regions, suggesting that activation of microglia was not caused by or resulted in neuronal death in the Mcoln1−/− brain.

**Abnormal synaptic plasticity in Mcoln1−/− mice**

Since our data show no overt neuronal loss in the Mcoln1−/− brain, we next examined the consequence of loss of TRPML1 on neuronal function, which might explain the severe motor and cognitive decline in MLIV. To assess synaptic function, we performed field recordings in the Schaeffer collateral pathway of acute hippocampal slices from seven month-old control and Mcoln1−/− mice (n = 6). First, we examined basal synaptic transmission by measuring the synaptic input–output relationship, plotting the slope of the field excitatory postsynaptic potential (fEPSP) as a function of the presynaptic fiber volley amplitude at varying stimulus intensities. Our results revealed a non-significant trend toward decreased synaptic input–output coupling in the Mcoln1−/− relative to control mice (p = 0.08) (Figure 4A). Second, we analyzed paired-pulse facilitation (PPF), a short-term form of presynaptic plasticity in which paired stimulation with a short inter-stimulus interval elicits a transient enhancement in neurotransmitter release. We observed significantly elevated PPF in the Mcoln1−/− hippocampus at the shortest
inter-stimulus intervals (ISI) (Figure 4B), implying that TRPML1 deficiency may affect neurotransmitter release. We also examined the impact of TRPML1 loss on synaptic plasticity by analyzing long-term potentiation (LTP), a long-lasting enhancement of synaptic strength induced by patterned high-frequency stimulation, which is thought to model synaptic modifications underlying learning and memory. Experiments in which LTP was induced by multiple trains of tetanic stimulation revealed that loss of TRPML1 led to significantly enhanced LTP (Figure 4C).
Alterations in synapse morphology in Mcoln1−/− mice

To determine if there were morphological changes associated with altered synaptic plasticity in Mcoln1−/− mice, we performed electron microscopic analysis of synaptic terminals in the CA1 stratum radiatum of 7 month-old mice. We observed elongated post-synaptic densities (PSD) in excitatory (asymmetric) synapses (p = 0.024) in Mcoln1−/− mice compared to control littermates (Figure 4D, E).

Quantification of the asymmetric synapse density in CA1 stratum radiatum showed a trend towards a decrease in Mcoln1−/− mice, but this did not reach statistical significance due to high variability (Figure 4F). Since axonal pathology is often accompanied by mitochondrial stress, we measured volume of mitochondria in neuropil of CA1 stratum radiatum in Mcoln1−/− and control mice, but found no difference (Figure 4G).

Loss of Mcoln1 results in the early defects of myelination

The most remarkable ultrastructural feature observed by electron microscopy in the stratum radiatum of Mcoln1−/− mice was reduced myelination (Figure 5A). Morphometric analysis revealed significant thinning of the myelin sheaths.
as shown by significantly lower g-ratio (axon diameter/fiber diameter, where fiber diameter is a sum of axon diameter and myelin sheath thickness) in Mcoln1−/− mice (Figure 5B). No significant difference was observed in the diameter of myelinated axons between the two genotypes (Figure 5C). Impaired CNS myelination and a dysgenic corpus callosum are hallmarks of neuropathology in MLIV patients, and a decreased Luxol fast blue staining has been previously reported in the corpus callosum, deep layers of neocortex and cerebellar white matter tracts of the end-stage Mcoln1−/− mice [26]. To follow the time-course of myelination deficits, we set out to measure myelination in two and seven month-old Mcoln1−/− mice using FluoroMyelin Green stain. Analysis of coronal sections (Figure 5D) revealed profound thinning of the corpus callosum and reduced myelination of internal capsule in Mcoln1−/− mice at both time points. Strikingly, we observed no anterior commissure (AC) in the Mcoln1−/− mice at two months. However, a dysmorphic and smaller AC appeared in the sections from
several month-old mice (Figure 5D). Analysis of the 
FlyoroMyelin Green staining in the sagittal sections re-
vealed malformation of the corpus callosum (CC) in the 
Mcoln1−/− mice. Specifically, similar to MLIV patients, 
Mcoln1−/− mice displayed a generally hypomorphic cor-
pus callosum, with dysgenic rostrum and dysgenic or 
absent splenium compared to WT littermate controls 
(Figure 5E).

Discussion
The most striking clinical manifestation of MLIV is psy-
chomotor retardation resulting in the developmental ar-
rest of motor, speech and cognitive function at the level of 
about 15 months. Thus, understanding the early patho-
logic events leading to neurologic and visual impairment 
is of prime importance for designing an effective therapy 
for MLIV. To study the first signs of neuropathology and 
to follow their maturation with disease progression, we 
performed open field testing on young animals and corre-
lated our findings with systematic volumetric and histo-
pathologic analysis and in vivo resting calcium imaging of 
the Mcoln1−/− mouse brain. Our results reveal that early 
motor and cognitive deficits in Mcoln1−/− mice are associ-
ated with white matter abnormalities, including hypogen-
esis of the corpus callosum, and pronounced activation of 
microglia and astrocytes, which is unexpectedly not ac-
companied by neurotoxicity or neuronal loss. However, 
we observe disrupted synaptic function, and increased 
length of asymmetric postsynaptic densities in excitatory 
synapses in the hippocampus of a MLIV mouse model at 
the late stage of disease.

Given the early onset of psycho-motor disabilities in 
patients with MLIV [5,37], we tested spontaneous loco-
motor and exploratory activity in juvenile one month-
old or two month-old young adult mice. Our results re-
vealed that the overall performance in the open field test 
changes with age, with a significant increase in the wild-
type littermates’ activity at two months of age compared 
to one month. However, while we observed no significant 
differences between the performance of one month-old 
wild-type and Mcoln1−/− mice, these mutant mice fail to 
undergo the normal developmental progression and showed 
significantly decreased jumping, rearing and exploratory ac-
tivity at two months compared to their control littermates, 
indicating an onset of the locomotor and potentially cog-
nitive decline due to the loss of TRPML1 at this age.

A striking early phenotype that we observed in Mcoln1−/− 
mice was activation of microglia and astrocytes. Activation of 
astrocytes has also been reported in the only brain 
autopsy case described in MLIV [9], where, based on 
general observations of reactive astrocytes accompanying 
neuronal death in other neurodegenerative conditions, it 
was thought to mark neuronal loss. Reactive astrocytes 
and microgliosis have been observed in mouse models 
of other LSDs, including the NCLs [27,38–43], mucopo-
lysaccharidosises [44]; Neimann-Pick disease [45] and 
mucolipidosis II [46]. In many of these models, neuroin-
flammation develops early and precedes neuronal loss 
in the gliosis-affected brain regions. However, our data 
show that in Mcoln1−/− mice gliosis was not accompa-
nied by neuronal loss even at the late stage of the disease.

To determine if the loss of TRPML1 or the activation 
of microglia or astrocytes observed in the Mcoln1−/− brain 
affects the physiological status of neurons, we measured 
esting concentrations of Ca2+ in neuronal somata and 
neuropil in the somatosensory cortex using in vivo multi-
photon microscopy with genetically encoded calcium indi-
cator YC3.60. The ability of neurons to maintain calcium 
homeostasis is a functional read-out of neuronal “health”. 
Overload of calcium leading to activation of calcineurin-
dependent neurodegenerative processes has been demon-
strated in the neurites of several mouse models of 
Alzheimer’s disease [28]. Moreover, in MLIV, intracel-
lar calcium dyshomeostasis due to loss of TRPML1, a 
lysosomal cation channel permeable to Ca2+, has been 
suggested in the literature as a primary mechanism of the 
disease [47,48]. Surprisingly, we observed no changes in 
esting Ca2+ concentration and virtually no effect of cyto-
solic storage material on intracellular [Ca2+] in Mcoln1−/− 
neurons of somatosensory cortex at the age of three 
months, implying spared physiologic status of neurons 
and absence of excitotoxicity in spite of TRPML1 loss and 
widespread reactive gliosis in the brain at this age.

Interestingly, in the hippocampus, activation of micro-
glia, which did not cause or result in the loss of CA1 or 
CA3 neurons, was accompanied by electrophysiological 
alterations (enhanced PPT and LTP) in the Schaeffer col-
laterals of the Mcoln1−/− mice. Of particular interest is 
the fact that re-expression of trpml in glial cells in the 
Drosophila model of MLIV was sufficient to restore sur-
vival, motor function and synaptic transmission [21]. 
This fact, together with the recent finding that activated 
microglia can facilitate the induction of LTP by releasing 
TNF [49], further highlights the role that glial cells may 
have in MLIV pathogenesis as well in other LSDs; a role 
which to date has been underappreciated. The mechan-
ism by which loss of TRPML1 causes a glial response is 
not clear. Recently TRPML1 was shown to be involved 
in the regulation of phagocytosis in macrophages [50] 
Further suggesting that neuroinflammation in MLIV could 
be caused by disturbed phagocytic activity in microglia. 
A more detailed study of the time course and mechanisms 
of neuroinflammation in Mcoln1−/− mice and primary cul-
tures of microglia and astrocytes will be in the focus of 
our future work.

Very little is known about brain pathology in human 
MLIV due to the lack of systematic clinical studies. An MRI 
study of 15 patients with MLIV revealed a characteristic
developmental impairment of the corpus callosum, white matter abnormalities on T1-weighted images and deposition of ferritin in basal ganglia and thalamus reflected by the changes in the signal intensities on T1- and T2-weighted MRIs [8]. In all but one MLIV patient with mild clinical manifestations, the corpus callosum was uniformly thinned, and had no rostrum together with an absent or dysplastic splenium. The thickness of corpus callosum in the patients with typical MLIV ranging from 16 months to 22 years of age varied from 2 to 3 mm which corresponds to the normal thickness at one month of age. These findings have been confirmed in the recent study recruiting five more MLIV patients of ages from 7 to 18 year-old [6]. Interestingly, we observed similar malformation of the corpus callosum with hypogenesis of the genu and trunk and characteristic agenesis of the rostrum and splenium in two month-old Mcoln1−/− mice. Developmental defects of the corpus callosum can be caused by impaired cell proliferation and migration, gliogenesis, axon growth, guidance or myelination. The mechanism resulting in dysgenic corpus callosum due to the loss of TRPML1 remains unclear. However, our data showing preserved cortical volume, thickness and neuron numbers in Mcoln1−/− mice make deficits in cell proliferation or neuron loss unlikely. Interestingly, a hypoplastic corpus callosum and other white matter abnormalities are found in many other LSDs: Gaucher, Krabbe, Pompe, Niemann-Pick, NCLs, mannosidosis, gangliosidoses and mucopolysaccharidosises (reviewed in [51]). These lysosomal diseases have an early onset in the first two years of life, a period critical for myelination, implying the role of aberrant lipid metabolism in oligodendrocytes in the pathogenesis of such disorders. Our data showing early myelination abnormalities in corpus callosum and other white matter structures such as internal capsule and anterior commissure and reduced thickness of myelin sheaths in the stratum radiatum of Mcoln1−/− mice further support this idea.

Our electrophysiological data showing enhanced PPF in the Schaefler collateral pathway are consistent with the previous report of decreased synaptic transmission in neuromuscular junctions in the Drosophila model of MLIV, which was shown to arise from the presynaptic impairment of synaptic vesicle cycling [21]. Notably, our results indicated a trend toward decreased synaptic transmission and decreased synapse density in MLIV mice, although these differences did not reach statistical significance. The significant increase in PSD length that we observed at excitatory synapses of CA1 neurons may represent a compensatory increase in postsynaptic active zone size in response to reduced synapsic input, or impaired internalization of AMPA-type glutamate receptors. Our data also raise the possibility that reduced myelination, the most remarkable ultrastructural finding, may contribute to the decreased presynaptic function we observed in MLIV mice. We suggest that the thinning of myelin sheaths in Schaefler collaterals may affect the efficiency of glutamate release through impaired propagation of action potentials and, thus, contribute to the development of cognitive impairment in MLIV. Massive axonal degeneration and formation of numerous axonal spheroids, which are full of aggregated mitochondria and vascular electron-dense structures, has been previously shown in cerebellar Purkinje neurons in the end stage Mcoln1−/− mice, indicating defects in axonal transport [26]. In contrast, we observed no such structures and no changes in the axon caliber in Schaefler collaterals, suggesting that pathway dependent axonal pathology occurs in these mice.

Overall, our data reveals that the glial activation is a dramatic and early feature of MLIV, and it is not associated with overt neurodegeneration in the course of disease. This finding changes our understanding of the disease mechanisms, shifting focus towards the role of lysosomes in glial cell health and functioning, and the role of glial dysfunction in the etiology of MLIV and other lysosomal diseases. It also opens new frontiers in therapy development to prevent or reverse the devastating neurologic symptoms of this disease. More specifically, along with traditional therapeutic approaches for LSDs involving substrate reduction or gene therapy, new paradigms such as glial progenitor cell replacement therapy or modulation of neuroinflammation can also be considered in MLIV.

Additional files

Additional file 1: Figure S1. Brain atrophy is absent in Mcoln1−/− mice in the course of disease. (A) Cavalieri estimates of the volume of hippocampus, cortex, thalamus and striatum obtained from wild-type (WT) and Mcoln1−/− (KO) littermates at two (n = 6 per genotype), three (n (WT) = 3; n (KO) = 4) and seven months of age (n = 4 per genotype). (B) Cortical thickness measured in the somatosensory barrelfield (S1BF), primary motor (M1), lateral entorhinal (LEnt) and primary visual (V1) areas. Two-way ANOVA (genotype x age) shows no significant interaction or significant effects of genotype at any of examined brain regions.

Additional file 2: Figure S2. Iba1 staining confirms microglia activation in Mcoln1−/− mice. Representative images of Iba1 immunostaining in wild-type (WT) and Mcoln1−/− (KO) littermates at two months of age showing increased immunoreactivity in KO in somatosensory barrelfield cortex. Scale bar is equal to 150 μm. Sections were counterstained with Gill Hematoxylin.

Additional file 3: Figure S3. Intragastric storage in CA1 stratum radiatum of Mcoln1−/− mice. (A) Representative electron micrograph showing accumulation of characteristic MLIV storage bodies with electron-dense granular and lamellar material (white arrowhead) in an astrocyte. (B) Accumulation of clusters of lysosome-like storage inclusions in a microglial cell or macrophage.

Additional file 4: Figure S4. Absence of neuronal loss in Mcoln1−/− mice. Optical fractionator estimates of number of neurons in the VPL-VPM, DLG and red nucleus of the thalamus, S1BF, and CA1 and CA3 subfields of the hippocampus in 7 month-old wild-type (WT) and Mcoln1−/− (KO) littermates (n = 4 per genotype). Data analyzed by t-test and show no significant differences between WT and KO in any of the examined brain regions.
Competing interests
The authors declare that they have no competing interests.

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