Mutations in CLCN6 as a Novel Genetic Cause of Neuronal Ceroid Lipofuscinosis

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Objective: The aim of this study was to explore the pathogenesis of CLCN6-related disease and to assess whether its Cl⁻/H⁺-exchange activity is crucial for the biological role of ClC-6.

Methods: We performed whole-exome sequencing on a girl with development delay, intractable epilepsy, behavioral abnormalities, retinal dysfunction, progressive brain atrophy, suggestive of neuronal ceroid lipofuscinoses (NCLs). We generated and analyzed the first knock-in mouse model of a patient variant (p.E200A) and compared it with a Clcn6⁻/⁻ mouse model. Additional functional tests were performed with heterologous expression of mutant ClC-6.

Results: We identified a de novo heterozygous p.E200A variant in the proband. Expression of disease-causing ClC-6E200A or ClC-6Y553C mutants blocked autophagic flux and activated transcription factors EB (TFEB) and E3 (TFE3), leading to autophagic vesicle and cholesterol accumulation. Such alterations were absent with a transport-deficient ClC-6E267A mutant. Clcn6E200A/⁻ mice developed severe neurodegeneration with typical features of NCLs. Mutant ClC-6E200A, but not loss of ClC-6 in Clcn6⁻/⁻ mice, increased lysosomal biogenesis by suppressing mTORC1-TFEB signaling, blocked autophagic flux through impairing lysosomal function, and increased apoptosis. Carbohydrate and lipid deposits accumulated in Clcn6E200A/⁻ brain, while only lipid storage was found in Clcn6⁻/⁻ brain. Lysosome dysfunction, autophagy defects, and gliosis were early pathogenic events preceding neuron loss.

Interpretation: CLCN6 is a novel genetic cause of NCLs, highlighting the importance of considering CLCN6 mutations in the diagnostic workup for molecularly undefined forms of NCLs. Uncoupling of Cl⁻ transport from H⁺ counter-transport in the E200A mutant has a dominant effect on the autophagic/lysosomal pathway.


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mRNA is expressed ubiquitously, whereas the CIC-6 protein is predominantly found in the nervous system where it resides on late endosomes. Clcn6−/− mice are viable, fertile, and have only mild behavioral abnormalities without neuron loss. However, they display a mild accumulation of lysosomal storage material in axon initial segments. The mechanism underlying this pathology and the precise physiological role of CIC-6 remains largely enigmatic. Unexpectedly, human genetics revealed an important, but poorly understood, role of CIC-6 in the brain. So far, 3 heterozygous disease-associated CLCN6 variants (p.E200A, p.Y553C, p.T520A) have been identified in patients with epileptic encephalopathy and early onset neurodegeneration, respectively. The severe neurological symptoms in individuals with heterozygous CLCN6 variants contrasts strikingly with the mild phenotype in Clcn6−/− mice and apparently unaffected heterozygous Clcn6+/− mice. This suggests that in patients, monoallelic variants of CLCN6 induce neurological defects through a gain, rather than loss-of-function. Indeed, the CIC-6Y553C and very recently the CIC-6T520A variants were shown to mediate current with larger amplitudes, likely because the voltage-dependence of its activation is shifted to less cytosolic-positive voltages.

CIC-6 is an electrogenic 2Cl−/H+ exchanger which mediates the exchange of Cl− against H+. The CLC family members share a critical glutamate residue in the ion permeation pathway that is involved in gating of CLC channels and in coupling Cl− to H+ fluxes of CLC exchangers, respectively. Like for other vesicular CLC exchangers, the mutation of the gating glutamate abolished CIC-6-mediated H+ transport, and converted the strongly voltage-dependent Cl−/H+ exchange of CIC-6 into a pure anion conductance. Recently, we reported a de novo heterozygous CLCN6 p.E200A variant of the critical gating glutamate in a patient with early-onset epilepsy, visual impairment, autism, and cognitive deficit. Equivalent uncoupling mutations in other CLC exchangers lead to pathology in mice models and human patients.

Here, we analyzed clinical and genetic characteristics of a 10-year-old girl with a de novo heterozygous p.E200A variant and generated the first knock-in mouse model harboring the uncoupling p.E200A mutation, to investigate the pathogenesis of CLCN6-related disease and to assess the relevance of Cl−/H+ exchange activity for the physiological role of CIC-6. The patient showed intractable epilepsy, behavioral abnormalities, retinal dysfunction, progressive brain atrophy, suggestive of neuronal ceroid lipofuscinoses (NCLs). Studies in cellular model systems showed that overexpression of disease-causing CIC-6 mutants impaired the lysosomal degradation of autophagic material, and influenced cholesterol metabolism. The mouse model harboring the heterozygous p.E200A variant phenocopies the CLCN6 p.E200A patients and exhibits characteristic features of NCLs. Our molecular analysis shows that disease-causing CLCN6 mutations result in defective mTORC1 signaling, specifically in the transcription factor EB (TFEB)/transcription factor E3 (TFE3) axis regulating autophagy and lysosome function.

Methods

Study Patient

The proband was referred to the Department of Pediatrics, Xiangya Hospital, Central South University, China. The study involving human subjects received approval from the Ethics Committee at Xiangya Hospital of Central South University (approval ID: #20170324) and was conducted in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent was obtained from the proband’s parents.

Whole Exome Sequencing (WES)

Peripheral blood was collected from the patient and her parents. WES sequencing was performed as previously described.

Animals

All animal experiments were performed according to the guidelines of Laboratory Animal Manual of the National Institute of Health Guide to the Care and Use of Animals, which were approved by the Institutional Animal Care and Use Committee of the Xiangya Hospital of Central South University (approval ID: #20170324). Mice were housed in the Central South University genetic animal facility or Delbrück-Centrum für Molekulare Medizin animal facility (approved by and in compliance with local authorities, LAGeSo, Berlin, Germany). Cln6E200A+/− knock-in and Cln6−/− knockout mice were generated by the Nanjing Biomedical Research Institute using CRISPR/Cas9 genome-editing technology. Details are described in the supporting information.

Histological and Histochemical Analyses

Histological and histochemical analyses are described in detail in the supporting information.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6. Statistical differences were analyzed using 1-way analysis of variance (ANOVA) or nonparametric, followed by Tukey’s multiple comparisons test. Data are represented as mean ± SEM. p-Values are depicted as: *p < 0.05; **p < 0.01; ***p < 0.001.
Results

Clinical Findings
The proband is a 10-year-old girl with severe developmental delay, intractable epilepsy, behavioral abnormalities, and microcephaly. A delay in P100 wave latency was observed in the visual evoked potential (Fig 1A). Brain MRI showed progressive cerebellar and cerebral atrophy co-occurring with enlargement of the lateral ventricles (Fig 1B). Electroencephalography (EEG) recordings revealed interictal bilateral sharp waves, spike waves, sharp and slow wave complexes, spike and slow wave complexes in bilateral frontal pole, frontal, anterior temporal region at 10 years (Fig 1C). WES and Sanger sequencing revealed that she has a heterozygous de novo CLCN6 c.599A > C, p.E200A variant (Fig 1D). With the proband, the total number of patients with CLCN6 disease-causing variants known to date is 6 (2 with the E200A variant, 3 with the p.Y553C variant, 1 with the p.T520A variant). They share some clinical features: global developmental delay, developmental regression, visual impairments, and brain imaging abnormalities, suggestive of NCLs. Further clinical details are described in the supporting information and Table S1.

Alterations of the Autophagic-Lysosomal Pathway (ALP) and TFEB/TFE3 Signaling and Accumulation of Free Cholesterol with Disease-Causing CIC-6 Mutants
To investigate the effects of CLCN6 mutations in the ALP, we expressed the p.E200A and p.Y553C patient mutants and the p.E267A mutant, which almost completely abolishes both Cl⁻ and H⁺-transport by CIC-6,9,10 in HeLa cells. We found that the microtubule-associated protein 1 light chain 3-II (LC3-II)/LC3-I ratio as well as protein levels of sequestosome 1 (SQSTM1/p62) were significantly increased upon expression of the p.E200A and p.Y553C mutants compared to wild-type (WT) CIC-6-transfected cells, whereas no such effect was found with the p.E267A mutant (Figs 2A and S1). These findings suggest an impairment of the ALP by the 2 disease-causing CIC-6 mutants, but not with the nearly transport-deficient p.E267A mutant.

Since TFEB and TFE3 are master regulators of ALP,16-18 we examined the nuclear translocation of enhanced green fluorescent protein (eGFP)-tagged TFEB and TFE3 in HeLa cells together with either WT or mutant CIC-6. Coexpression with the mutants p.E200A or p.Y553C, but not p.E267A, resulted in a drastic increase of nuclear eGFP-TFEB and eGFP-TFE3 (Fig 2B,C), compared with WT CIC-6 coexpression. These observations indicate an activation of TFEB and TFE3 signaling by the presence of the disease-causing CIC-6 mutants.

In order to test whether further functions of lysosomes are impaired, we assessed free cholesterol levels by Filipin staining, and found more Filipin staining in cells expressing CIC-6E200A or CIC-6Y553C mutants than in cells expressing WT CIC-6 or CIC-6E267A mutant (Figs 2D and S2). These results suggest an accumulation of free cholesterol upon expression of disease-causing mutant CIC-6.

Neurological Phenotypes of Clcn6E200A+/Mice
Clcn6E200A+/ and Clcn6−/− mice were generated using CRISPR/Cas9 technology (Fig S3). As we failed to mate heterozygous Clcn6E200A+/ with WT mice, sperm of male Clcn6E200A+/ mice were used for in vitro fertilization. Clcn6E200A+/ and Clcn6−/− mice were born with Mendelian ratio. However, the survival rate of Clcn6E200A+/ mice dropped markedly by 4 months. All Clcn6E200A+/ mice died within 7 months (Fig 3A), accompanied by a weight loss after 24 weeks (Fig 3B). Furthermore, Clcn6E200A+/ mice manifested neurological deterioration attested by abnormal limb-clasping reflexes by 8 weeks (Fig 3C). Disease symptoms progressed rapidly with the onset of a tremor at 3 months that became progressively more severe and was accompanied by locomotor difficulties including hunched gait and ataxia (Movies S1 and S2). In contrast, as reported for a previously generated mouse line,3 homozygous Clcn6−/− and heterozygous Clcn6+/− mice were indistinguishable in this respect from their WT littermates and had a normal lifespan.

In p.E200A patients, brain MRI shows brain atrophy (this study and ref4). Similarly, high-resolution MRI revealed a significant decrease in brain volume and enlarged ventricles of 5-month-old Clcn6E200A+/ mice (Fig 3D). Remarkably, although our tests revealed no neurological phenotypes in Clcn6−/− mice, similar dilation of lateral ventricles was observed (Fig 3D).

Like for the p.E200A patients (this study and ref4), seizures were observed in 4- to 5-month-old Clcn6E200A+/ mice, but not in Clcn6−/− mice (Movies S3–S6). Importantly, video EEG recordings showed massive spike-wave and rhythmic spikes discharges in Clcn6E200A+/ mice, whereas only few low-amplitude spike waves were observed in age-matched Clcn6−/− mice (Fig 3E). We did not observe a direct link between the seizures and the death of the mice as recently shown for mice with a pathogenic Cln2 variant.19

To test for retinal dysfunction, as found for the p.E200A patients (this study and ref4) we performed electroretinographic (ERG) analyses. The b-wave response
FIGURE 1: Clinical data of the patient. (A) Visual evoked potential test at 10 years showed a delay in P100 wave latency. (B) Brain MRI (T1-weighted, sagittal, and coronal planes) of the patient at the age of 4 years (top row), the age of 8 years (middle row), and the age of 10 years (bottom row) showing progressive cerebellar and cerebral atrophy with a secondary hydrocephalus. (C) Video EEG at 10 years of age showing interictal bilateral sharp waves, spike waves, sharp and slow wave complexes, spike and slow wave complexes were evident in bilateral frontal pole, frontal, anterior temporal region. (D) Sanger sequence chromatograms showing a de novo heterozygous constitutive c.599A > C in CLCN6. [Color figure can be viewed at www.annalsofneurology.org]
amplitudes were dramatically reduced in all dark and light adapted tests, as well as both scotopic and photopic a-wave response amplitudes at any stimulus intensity level, with the only exception at a flash strength of 3 cd.s/m² in 4–6 months old Clcn6E200A/+ mice (Fig 3F). In Clcn61−/− mice, the scotopic b-wave amplitudes also decreased significantly, but more mildly at all level stimulus intensities, and the photopic b-wave responses were much weaker compared to WT controls at 10 and 30 cd.s/m² (Fig 3F).

These data indicate retinal dysfunction in both Clcn6E200A/+ and Clcn61−/− mice. However, hematoxylin and eosin (H&E) staining revealed no retina degeneration in 5-month-old Clcn6E200A/+ or, as previously shown, Clcn61−/− mice (Fig S4).

**Behavioral Abnormalities of Clcn6E200A/+ Mice**

Next, we performed a battery of behavioral assays with 1, 3, and 5 months old WT, Clcn6E200A/+ and Clcn61−/−...
FIGURE 3: Phenotypes of Clcn6<sup>E200A</sup>/<sup>+</sup> and Clcn6<sup>−/−</sup> mice. (A) Survival analysis indicated reduced lifespan of Clcn6<sup>E200A</sup>/<sup>+</sup> mice. n ≥ 15 per genotype. (B) Body mass was reduced in Clcn6<sup>E200A</sup>/<sup>+</sup> mice at 24 weeks (near the terminal stage), but not in Clcn6<sup>−/−</sup> mice. n ≥ 8 per genotype. (C) Clcn6<sup>E200A</sup>/<sup>+</sup> mice, but not Clcn6<sup>−/−</sup> mice manifested clasping behavior. n ≥ 12 per genotype. (D) The 5-month-old Clcn6<sup>E200A</sup>/<sup>+</sup> mice displayed a significant decrease of brain volume, and enlarged ventricles. Similar dilation of lateral ventricles, but no changes in brain volume was observed in 5-month-old Clcn6<sup>−/−</sup> mice as assessed by brain MRI. Scale bar: 20 mm. (E) EEG recordings showed massive spike–wave and rhythmic spike discharges in 4-to-5-month-old Clcn6<sup>E200A</sup>/<sup>+</sup> mice, whereas only a few low-amplitude spike waves were seen in age-matched Clcn6<sup>−/−</sup> mice. (F) ERG recordings showed both a- and b-waves were markedly reduced in Clcn6<sup>E200A</sup>/<sup>+</sup> mice. [Color figure can be viewed at www.annalsofneurology.org]
mice. Balance beam and rotarod tests revealed impaired motor coordination at all ages in Clcn6<sup>E200A/+</sup> mice, characterized by a longer latency to cross the beam and a shorter latency to fall off the rod starting at 1 month, and higher number of foot slips starting at 3 months (Figs S5A and S5B). Likewise, we also observed an abnormal gait with markedly wide-based stance, small stride, and separated hindlimb prints in Clcn6<sup>E200A/+</sup> mice (Fig S5C). At 5 months, Clcn6<sup>E200A/+</sup> mice scored drastically lower than WT controls in both coat-hanger and hanging wire tests (Figs S5D and S5E), indicating decreased muscle strength. In the open field test, Clcn6<sup>E200A/+</sup> mice showed a longer total track length at 3 months, whereas they traveled significantly less spontaneously at 5 months compared to age-matched WT controls (Fig S5F), indicating hyperactivity in the early stage and a decreased exploratory activity at the end stage. Dwelling time in center field was strongly decreased at 3 months (Fig S5F), consistent with anxiety-like phenotypes. In contrast, Clcn6<sup>−/−</sup> mice behaved similarly to WT controls in all the tests except for the open field assay, where they exhibited anxiety-like phenotypes at 5 months (Fig S5A–F).

**Progressive Neurodegeneration and Early Glial Responses in Clcn6<sup>E200A/+</sup> Brain**

As MRI showed brain atrophy in the Clcn6<sup>E200A/+</sup> mice, we measured brain weights among the 3 genotypes. Clcn6<sup>E200A/+</sup> mice possessed significantly smaller brains with reduced weight compared to WT littermates or Clcn6<sup>−/−</sup> counterparts at 4 months, but not at P30 (Figs 4A and S6). As there was no significant difference in body weight between the genotypes at these ages, these results point toward a brain degeneration in Clcn6<sup>E200A/+</sup> mice. In addition, cortex thickness measurement revealed widespread atrophy of the cortex in 5-month-old Clcn6<sup>E200A/+</sup> mice that was not evident in age-matched Clcn6<sup>−/−</sup> mice (Fig. S7). Therefore, we examined the onset and progression of neuropathological changes systematically in Clcn6<sup>E200A/+</sup> and Clcn6<sup>−/−</sup> mice at postnatal days P30, P60, P90, P120, and P150. We found that neuron loss in Clcn6<sup>E200A/+</sup> mice became firstly apparent in the cornu ammonis (CA) 3 region of the hippocampus at P60 (Fig 4B). Loss of Purkinje cells was seen as early as P90 (Fig 4C), in agreement with the development of tremor and ataxia around this time. Specifically, the loss of Purkinje cells started preferentially, and was particularly pronounced in lobule I and lobule II of the cerebellum. At the end stage, immunohistochemical staining for the Purkinje cell marker calbindin revealed almost complete loss of Purkinje cells, except for lobules IX and X, in Clcn6<sup>E200A/+</sup> mice (Fig S8). On the other hand, histological staining of Clcn6<sup>−/−</sup> brain sections revealed no morphological abnormalities up to 20 months (Figs 4C and S9). Assessment of survival of interneurons in the cortex and hippocampus of Clcn6<sup>E200A/+</sup> mice revealed loss of somatostatin-positive (SS1+) neurons, but not of parvalbumin-positive (PV+) or calretinin-positive (CR+) neurons (Figs S10–S12). Luxol-fast blue staining revealed a clear disruption of myelin in the brain of 5-month-old Clcn6<sup>E200A/+</sup> mice and mildly disturbed myelination in Clcn6<sup>−/−</sup> mice (Fig 4D). Together, these data demonstrated brain atrophy and degeneration in Clcn6<sup>E200A/+</sup> mice.

Neurodegeneration was paralleled by an activation of astrocytes and microglia, as revealed by astrocyte marker glial fibrillary acidic protein (GFAP) and microglia marker ionized calcium binding adaptor molecule 1 (IBA-1) staining of the brain from 4-to-5-month-old Clcn6<sup>E200A/+</sup> mice (Figs 4E, S13 and S14) and immunoblotting (Fig S15). To test whether glial activation precedes or follows neuron loss, we examined astrocytes and microglia in symptomatic P30 Clcn6<sup>E200A/+</sup> mice, when degeneration is observed in neither these, nor in Clcn6<sup>−/−</sup> mice (Fig 4F). In P30 Clcn6<sup>E200A/+</sup> mice, GFAP and IBA-1 immunoreactivity was prominent and morphologically similar to that observed at 4 months, but was less markedly different from WT controls (Figs 4F and S16–S19). On the other hand, Clcn6<sup>−/−</sup> brain sections revealed no astrogliosis or microgliosis at any age tested (Figs 4E,F and S13–S17). Taken together, Clcn6<sup>E200A/+</sup> mice exhibit early and progressive astrocytic and microglial reactive changes, events occurring before obvious neuron loss.

**Accumulation of Autofluorescent Lysosomal Storage Material in Clcn6<sup>E200A/+</sup> Neurons**

We observed widespread intracellular accumulation of autofluorescent storage material in many brain regions of Clcn6<sup>E200A/+</sup> mice at 5 months (Fig 5A). Interestingly, while storage material was present predominantly in the proximal axon in Clcn6<sup>−/−</sup> mice as shown previously,<sup>3</sup> it was scattered in punctate structures over the neuronal soma and axon of Clcn6<sup>E200A/+</sup> mice.

To examine the morphology of the deposits in more detail, we performed electron microscopy of cortex, hippocampus, and cerebellum. This revealed abnormal electron-dense lysosomal storage material in the somata of Clcn6<sup>E200A/+</sup> neurons and in the proximal axon of Clcn6<sup>−/−</sup> neurons at 5 months (Fig 5B). Higher magnification of deposits showed an accumulation of lysosomes, autophagosomes, numerous intracellular inclusion bodies with double membranes or multilamellated electron-dense material, damaged organelles, lipid droplets,
amorphous or granular lipofuscin deposits in the neurons of Clcn6^{E200A/+} mice (Fig 5C–L), whereas Clcn6^{−/−} neurons only showed lipid droplets associated with electron-dense amorphous or granular lipofuscin deposits in the proximal axons (Fig 5M,N). The aberrant accumulation of organelles and storage material in neurons of Clcn6^{E200A/+} mice is a characteristic feature of NCLs. In addition, autophagosomes and lysosomes were significantly larger in neurons of Clcn6^{E200A/+} compared to WT mice (Fig 5), which suggest that autophagy and lysosomal degradation are disturbed in Clcn6^{E200A/+} mice.
FIGURE 5: Accumulation of neuronal autofluorescent lysosomal storage material in Clcn6<sup>E200A</sup><sup>+/+</sup> mice. (A) Representative images of the frontal cortex in unstained brain sections visualized at 543 ± 11 nm excitation and 593 ± 20 nm detection show the abundant intracellular accumulation of autofluorescent storage material in neuronal soma and axon of 5-month-old Clcn6<sup>E200A</sup><sup>+/+</sup> mice compared with age-matched WT controls. While autofluorescence is visible in the proximal axon of 5-month-old Clcn6<sup>−/−</sup> mice. (B) Electron micrographs showed massive electron-dense lysosomal storage material in the perikaryal of Clcn6<sup>E200A</sup><sup>+/+</sup> neurons (representative images of the frontal cortex), which is less abundant in the neurons of Clcn6<sup>−/−</sup> mice and only locates in the proximal axon of Clcn6<sup>−/−</sup> neurons. (C-L) Higher magnification shows an accumulation of lysosomes (blue asterisks), double membrane autophagosomes (yellow arrows), numerous intracellular inclusion bodies (blue arrows) with double membranes or multilamellated electron-dense material (red arrowhead), damaged organelles, lipid droplets (red asterisks), amorphous or granular lipofuscin deposits (red arrows) in neurons of Clcn6<sup>E200A</sup><sup>+/+</sup> mice. (M,N) Higher magnification demonstrates only lipid droplets associated with electron-dense amorphous or granular lipofuscin deposits in neurons of Clcn6<sup>−/−</sup> mice. No lysosomes, autophagosomes, intracellular inclusion bodies, or damaged organelles were observed. MT = mitochondrion, ER = endoplasmic reticulum, GN = Golgi. Scale bars: 20 μm (A), 2 μm (B), 200 nm (C-N).
Metabolic Disorder in Clcn6<sup>E200A/+</sup> Brain

To assess brain carbohydrate accumulation in Clcn6<sup>E200A/+</sup> and Clcn6<sup>−/−</sup> mice, we performed periodic acid Schiff (PAS) staining in 5-month-old WT, Clcn6<sup>E200A/+</sup> and Clcn6<sup>−/−</sup> mice. We observed PAS-positive granular deposits not only in the perikaryon of neurons, but also in the intercellular space of cortex, hippocampus, and cerebellum of Clcn6<sup>E200A/+</sup> mice (Fig 6A). By contrast, in Clcn6<sup>−/−</sup> mice, no positive labeling with PAS staining was detectable (Fig 6A). Next, we measured lipids in the brain of WT,

FIGURE 6: Metabolic disorder in the brain of Clcn6<sup>E200A/+</sup> and Clcn6<sup>−/−</sup> mice. (A) PAS staining of paraffin-embedded brain sections revealed accumulation of carbohydrates in the intercellular space of the cortex (representative images of the primary motor cortex) and cerebellum, and in the neuronal perikaryon of the hippocampus of 5-month-old Clcn6<sup>E200A/+</sup> mice, but not in Clcn6<sup>−/−</sup> mice. (B) Luxol fast blue staining for lipofuscin revealed granular deposits in most neuronal somata, particularly in Purkinje and granule cells of the cerebellum, of 5-month-old Clcn6<sup>E200A/+</sup> and Clcn6<sup>−/−</sup> mice. For the cortex, representative images of the primary motor cortex are shown. (C) Oil Red O staining revealed the existence of massive red-stained lipids in neurons of the cortex (representative images of the secondary visual cortex, mediolateral), hippocampus and cerebellum of both 5-month-old Clcn6<sup>E200A/+</sup> and Clcn6<sup>−/−</sup> mice. (D) Filipin staining showed more unesterified cholesterol in the cortex (representative images of the primary motor cortex), hippocampus and cerebellum of 5-month-old Clcn6<sup>E200A/+</sup> mice than in age-matched WT or Clcn6<sup>−/−</sup> mice. Scale bars: 10 μm.
Granular deposits in most neuronal somata, particularly in Purkinje and granule cells of the cerebellum, of 5-month-old Clcn6E200A+/+ and Clcn6E200A−/− (Fig 6B). Additionally, Oil Red O staining revealed massive lipid accumulation in neurons of the cortex, hippocampus, cerebellum of both 5-month-old Clcn6E200A+/+ and Clcn6E200A−/− mice (Fig 6C). As expected, and consistent with our in vitro data (Fig 2D), 5-month-old Clcn6E200A+/+ mice had more unesterified cholesterol in the cortex, hippocampus, cerebellum than age-matched WT or Clcn6E200A−/− mice, as shown by Filipin staining (Fig 6D). Taken together, these results demonstrate that glycometabolism and lipid metabolism disorder were evident in Clcn6E200A+/+ brain, while only lipid metabolism was disrupted in Clcn6E200A−/− brain.

Increased Protein Levels of CIC-6E200A+, But Not of Other Vesicular CLCs in Clcn6E200A+/+ Brain

For CIC-6, we found remarkably increased protein levels in all examined brain regions of P120 Clcn6E200A+/+ mice compared to WT mice (Figs 7A and S20). Notably, for newborn mice (postnatal day 0 [P0]), no significant differences in CIC-6 levels were observed between WT and Clcn6E200A+/+ brain (Fig S21), which shows that accumulation of mutant CIC-6 protein occurs only later. As expected, CIC-6 protein was not detectable in liver of each genotype, or in brain tissues of Clcn6E200A−/− mice (Figs 7A, S20 and S21). The increase of CIC-6 levels in the brain of 4-month-old Clcn6E200A+/+ mice was not due to increased gene transcription as quantitative real-time polymerase chain reaction (qRT-PCR) revealed unchanged Clcn6 mRNA levels compared with WT controls (Fig S22).

The strikingly more severe phenotype in Clcn6E200A+/+ mice compared to Clcn6E200A−/− mice could be explained by the possibility that other vesicular CLC proteins might compensate for a loss of CIC-6 function in Clcn6E200A−/−, but not in Clcn6E200A+/+ mice. However, immunoblot examination of the protein levels of the CIC-6 paralogs expressed in brain revealed unchanged protein levels of CIC-3, CIC-4 and CIC-7 in Clcn6E200A+/+ and Clcn6E200A−/− P0 brains (Fig S21).

Early and Progressive Lysosomal Impairments in Clcn6E200A+/+ Brain

To evaluate lysosomal function, we first analyzed the expression levels of cathepsin D (CTSD) and lysosomal-associated membrane protein 1 (Lamp1). Compared to WT control brains, the levels of pro-CTSD and Lamp1 were increased in Clcn6E200A+/+ mice at P35 (Fig S23), a time when the overall cellular morphology appeared normal but neurons were already dysfunctional, and further increased in all tested brain areas at P120 Clcn6E200A+/+ brain (Figs 7A and S20). We also observed increased levels of mature-CTSD in all tested brain areas at P120 Clcn6E200A+/+ brain (Figs 7A and S20). However, we did not find any differences among the 3 genotypes at asymptomatic age (P0) (Figs 7B and S24). These abnormalities in CTSD and Lamp1 levels were not observed in Clcn6E200A−/− mice at all ages tested, regardless of the brain regions examined (Figs 7A,B, S20, S23, and S24). As expected, CTSD and Lamp1 levels were not altered in the liver of P120 Clcn6E200A+/+ and Clcn6E200A−/− mice (Figs 7A,B and S20). Immunohistochemistry confirmed the increase of CTSD in cortex, hippocampus, and cerebellum of 4-month-old Clcn6E200A+/+ mice (Fig 7C). While total protein levels of CTSD were unchanged in P120 Clcn6E200A−/− brain, immunohistochemistry revealed that CTSD was concentrated in proximal axons and strongly reduced in somata of 4-month-old Clcn6E200A−/− brain (Fig 7C). However, the enzymatic activity of CTSD was not significantly altered in the cortex of Clcn6E200A+/+ or Clcn6E200A−/− mice at 5 months (Fig S25). Overall, these results hint at an early and progressive impairment in lysosomal function in the Clcn6E200A+/+ brain.

Early and Progressive Autophagy Defects in Clcn6E200A+/+ Brain

Consistent with the effect of ectopically expressed CIC-6 variants in HeLa cells (Figs 2A and S1), the LC3-II/LC3-I ratio was significantly increased in the brain of Clcn6E200A+/+ mice at P35 (Fig S23) in comparison to WT, and further increased in all tested brain areas of Clcn6E200A+/+ mice at P120 (Figs 7A, and S20). This was accompanied by a significant increase in the autophagy substrate protein SQSTM/p62 (Figs 7A and S20 and S23). In contrast, the LC3-II/LC3-I ratio and the SQSTM1/p62 levels were unchanged at P0 (Figs 7B and S24). Altogether, the gradual accumulation of LC3-II and SQSTM1/p62 suggests a lysosomal dysfunction in neurons of Clcn6E200A+/+ mice leading to an accumulation of autophagosomes. Conversely, there was no substantial difference in the LC3-II/LC3-I ratio or the SQSTM1/p62 levels between Clcn6E200A−/− and WT brains at any age (Figs 7A,B, S20, S23, and S24). In addition, immunohistochemistry analyses showed strong staining of SQSTM1/p62 in many brain regions of Clcn6E200A+/+ mice at 4 months, but not age-matched WT or Clcn6E200A−/− mice (Fig 7D). Moreover, we observed higher levels of mitochondrial ATP synthase subunit c (SCMAS) immunoreactivity in various brain regions of P100 Clcn6E200A+/+ mice than in WT controls, whereas SCMAS immunoreactivity in the Clcn6E200A−/− brain was concentrated in proximal axons (Fig 7E). Consistent with the immunohistochemistry
FIGURE 7: Lysosomal function and autophagy in Clcn6<sup>E200A/+</sup> mice. (A) Western blot analysis showed an increase of CIC-6, Lamp1, pro-CTSD, mature-CTSD, SQSTM1/p62, and LC3-II/ LC3-I in the cortex, hippocampus, cerebellum, white matter, and brainstem from P120 Clcn6<sup>E200A/+</sup> mice compared to WT littermates. For quantification, see Figure S20. (B) No change of CIC-6, Lamp1, pro-CTSD, mature-CTSD, SQSTM1/p62, and LC3-II/ LC3-I in total brain extracts of Clcn6<sup>E200A/+</sup> mice, compared to WT littermates at P0. For quantification, see Figure S24. (C) Intense immunostaining for CTSD was localized to neuronal perikarya in 4-month-old Clcn6<sup>E200A/+</sup> mice, whereas mild immunoreactivity is only detected in the proximal axon of age matched Clcn6<sup>−/−</sup> mice. (D) Immunohistochemical staining showing SQSTM1/p62-positive aggregates in the cortex, hippocampus, and cerebellum from 5-month-old Clcn6<sup>E200A/+</sup> mice, compared to age-matched WT or Clcn6<sup>−/−</sup> mice. (E) Immunostaining for SCMAS is large-granular and intensely localized to neuronal perikarya in P100 Clcn6<sup>E200A/+</sup> mice, whereas mild immunoreactivity is only detected in the proximal axon of P100 Clcn6<sup>−/−</sup> mice. (C-E) For the cortex, representative images of the primary motor cortex are shown. Scale bars: 10 μm.
studies, Western blot analysis showed an increase of SCMAS in total brain extracts of Clcn6\textsuperscript{E200A/+} mice at 5 months, compared to age-matched WT or Clcn6\textsuperscript{+/+} mice (Fig S26). These data imply impairment of autophagy of mitochondria (mitophagy) in the brain of Clcn6\textsuperscript{E200A/+} mice. Collectively, our data show that autophagy defects are early pathogenic events in the Clcn6\textsuperscript{E200A/+} brain preceding neurodegeneration.

**Altered mTOR-TFEB Signaling in Clcn6\textsuperscript{E200A/+} Brain**

Overexpression of ClC-6\textsuperscript{E200A} or ClC-6\textsuperscript{Y553C} activated TFEB (Fig 2B). Therefore, we assessed the expression of TFEB target genes, including autophagy (Lc3b, Sqtmm1/p62, Vps8, Vps11, and Uvrag) and lysosomal (Lamp1, Cln3, Wipi, Ctsa, Ctsb, Ctsd, Gisf, Atp6v1h, and Clcn7) genes in the brains of 4-to-5-month-old WT, Clcn6\textsuperscript{E200A/+}...
and Clcn6+/− mice. qRT-PCR revealed an increase in mRNA levels of many lysosomal genes, including some orthologs of human NCL genes (CLN3, CLN10/CTSD, and CLN13/CTSF), and of some autophagic genes (Lc3Bb, Sqtnt1p62) in Clcn6E200A+/+ mice compared to their WT littermates (Fig 8A). In contrast, there was no difference in the mRNA levels of lysosomal or autophagic genes in brain of Clcn6+/− mice (Fig 8A). Consistent with increased lysosomal biogenesis in the presence of CIC-6E200A, we observed a significant increase in the number and size of LysoTracker-stained acidic vesicles in cells ectopically expressing CIC-6E200A, compared to WT CIC-6-transfected cells (Figs 8B and S27).

As TFEB activity is regulated by the kinase mTORC1, we tested for mTORC1 activity. We found significantly reduced levels of phosphorylated p70S6K, a major mTORC1 target, in brain extracts of 4-month-old Clcn6E200A+/+ mice, compared to those of WT and Clcn6+/− mice (Figs 8C and S28). The reduction in mTORC1 activity was not due to decreased protein level as no change in mTOR protein levels was detected in Clcn6E200A+/+ brain (Figs 8C and S28).

Taken together, these data suggest that the activity of mTORC1 is reduced in Clcn6E200A+/+, leading to increased TFEB activity, which in turn results in increased lysosomal size and number.

Neuronal Apoptosis in Clcn6E200A+/+ Brain

Last, we tested for an involvement of apoptosis in the neuronal degeneration in Clcn6E200A+/+ mice. Procaspase-3 and cleaved caspase-3 were markedly elevated in 4-month-old Clcn6E200A+/+ mice, compared to WT or Clcn6+/− counterparts (Figs 8C and S28). However, procaspase-3 and cleaved caspase-3 showed no differences among the 3 genotypes at P35 (Figs 8D and S29). These results indicate that apoptosis occurs only at later stages in Clcn6E200A+/+ mice.

Discussion

In this study, we describe a girl with a de novo CLCN6 p.E200A variant, who presented intractable epilepsy, progressive cognitive decline, motor impairment, behavioral abnormalities, retinal dysfunction, and progressive brain atrophy, suggestive of NCLs. Moreover, we characterized the first Clcn6 knock-in mouse model harboring a heterozygous disease-associated Clcn6 variant. Heterozygous Clcn6E200A+/− mice developed severe neurological phenotypes including tremor, seizures, ataxia, visual impairment, behavioral abnormalities, and premature death, all together resembling human NCLs.21–23 Clcn6E200A+/− mice also developed a number of neuropathological features similar to those that occur in NCLs,21,22 including a massive accumulation of SCMAS and autofluorescent lipopigment, brain atrophy, progressive neuron loss, defective myelination as well as progressive glial activation. So, our study shows that the Clcn6E200A+/− mutant mouse model displays many phenotypic similarities to the patient with the equivalent CLCN6 variant, and recapitulates virtually all characteristic clinical and pathological features of human NCLs.

NCLs are a devastating subclass of genetic lysosomal storage diseases (LSDs) which manifest in early childhood.21 So far, 13 different NCL genes (CLN1–8,10–14) have been identified, many of which encode proteins in the endosomal/lysosomal pathway.22–26 The existence of patients with NCL-like pathology but no mutation in any of these genes suggests additional unknown NCL genes.27 Patients with the heterozygous CLCN6 p.E200A variant as well as 3 patients carrying a heterozygous CLCN6 p.Y553C variant and 1 patient with the p.T520A variant showed progressive cognitive decline, motor deficiencies, visual impairment, and developmental regression,4,6,8 symptoms strikingly resembling those seen in NCL patients. Expression of CIC-6E200A or CIC-6Y553C mutants in HeLa cells impaired the lysosomal degradation of autophagic material, and led to free cholesterol accumulation, indicating disease-causing CIC-6 mutants cause a kind of metabolic disorders, such as LSDs. Our Clcn6E200A+/+ mice phenocopied the CLCN6 p.E200A patients, and the Clcn6E200A+/+ brain pathology mirrored the pathology of human NCLs. Altogether, this study demonstrates that CLCN6 is a novel gene for NCLs and indicates that CLCN6 should be considered in the diagnostic workup for molecularly undefined forms of NCLs.

There is a striking difference between the clinical features and symptoms found in human with CLCN6 disease-causing mutations and Clcn6E200A+/+ mice on the one side and the much milder phenotype in Clcn6+/− mice,3 which was reproduced in this study, on the other side (Table S2). Clcn6+/− mice only displayed mild behavioral abnormalities from 3 to 5 months of age.3 Their brains showed no prominent brain atrophy or obvious neuron loss, despite marked autofluorescence in proximal axons, which were often ballooned and contained lysosomal storage material.3 Unexpectedly, our analyses showed previously unrecognized ventricle dilation and retinal dysfunction in Clcn6+/− mice. Nonetheless, the overall absence of an obvious neurological phenotype and neuron loss in Clcn6+/− mice demonstrates that CIC-6 loss-of-function only mildly impinges on neuronal function. In addition, the CIC-6E200A mutant suppressed mTORC1 activity, enhanced TFEB and TFE3 translocation and up-regulated expression of autophagic-lysosomal genes, whereas deletion of CIC-6 did not cause such changes. Therefore, these data suggest that the p.E200A
mutation causes toxicity by a gain-of-function, similar to the p.Y553C mutation and consistent with the dominant phenotype in the heterozygous patients and animals. The p.Y553C mutant mediates larger currents especially at lower extracellular pH. A gain-of-function by the p.E200A mutation, despite its uncoupling of anion transport from proton antiport, may be explained by the loss of rectification that allows Cl− transport at voltages where the rectifying exchanger mediates less current.

The severe consequences of the heterozygous p.E200A variant in the patients and in the mouse model are also intriguing in respect to the equivalent mouse models for CLC paralogs. Mutations in all of the neuronal CLC Cl−/H+ exchangers CIC-3, −4, −6, and −7 underlie a spectrum of neurological disorders.

Mouse models lacking the endosomal CIC-3 or the lysosomal CIC-7 develop neurodegeneration. The complete substitution of the respective Cl−/H+ exchanger, in the background of CIC-4-deficiency in the case of CIC-3, by its gating glutamate mutant that uncouples chloride from proton transport results in virtually the same phenotype as its knock-out. However, mice heterozygous for such mutation in Cln3 or Cln7 do not present obvious phenotypes. Likewise, mice and patients with uncoupled CIC-5 display virtually the same phenotype as observed with a loss of CIC-5 function. It remains to be determined why the gating glutamate mutation in CIC-6 has a dominant effect that is not found in the other vesicular CLCs.

Electron microscopy studies showed an accumulation of lipid droplets and lipofuscin in the neuronal soma and axon of Cln6+/E200A+ mice, whereas this storage material was present predominantly in the proximal axon of Cln6−/− mice. In histochimistry, the storage material in Cln6+/E200A+ brain was strongly autofluorescent, positive for Luxol-fast blue, Oil red O, and Filipin, and distributed in the perikaryon. In contrast, the storage material in Cln6−/− brain was strongly autofluorescent, only positive for Luxol-fast blue, Oil red O, but negative for Filipin. Lipidomic studies are needed to determine the exact nature of the lipids stored in the Cln6+/E200A+ and Cln6−/− neurons. In addition, we observed PAS-positive granular deposits in the perikaryon of neurons, and in the intercellular space of cortex, hippocampus, and cerebellum of 5-month-old Cln6+/E200A+ mice, whereas such PAS staining was not detected in age-matched Cln6−/− brain. Collectively, these data suggest that a lipid metabolism disorder may contribute to the common neuropathology in both Cln6+/E200A+ and Cln6−/− mice, which ultimately leads to myelination defects. By contrast, the disturbance of glycometabolism may be associated with more severe neuropathology in Cln6+/E200A+ mice. Therefore, regulating lipid metabolism and glycometabolism may be a therapeutic option for the treatment of CLCN6-related NCLs.

The autophagic-lysosomal pathway is a critical cellular quality control system. Its disturbance has been recognized as a major pathomechanism contributing to the accumulation of storage material and neurodegeneration in various neurodegenerative diseases including several NCLs. The accumulation of autophagic vacuoles and the age-dependent increase in autophagic marker proteins suggest that the autophagic degradation pathway is impaired in the brain of Cln6+/E200A+ mice. While not detectable in newborn mice, autophagic defects developed with the time of lysosomal dysfunction, in agreement with a contribution of autophagy defects to the initiation of neuron loss in Cln6+/E200A+ mice.

The accumulation of autophagic vacuoles in Cln6+/E200A+ mice is likely due to impaired clearance secondary to lysosomal dysfunction, as we previously found reduced autophagosome-lysosome fusion in CIC-6+/E200A− expressing cells. Accumulation of storage material upon lysosomal dysfunction, which we find in Cln6+/E200A+ mice, triggers mTORC1-TFEB/TFE3 signaling leading to enhanced lysosomal biogenesis. Consistently, mTORC1 activity was decreased in mouse brains and various lysosomal proteins. Their mRNA levels showed an age-dependent increase in Cln6+/E200A− mouse brain. Overexpression of disease-causing p.E200A and p.Y553C CIC-6 mutants induced TFEB and TFE3 nuclear translocation in HeLa cells as well. Increased Lamp1 and CTSD levels were detected in P35 Cln6+/E200A− brains, many days before obvious neuron loss, indicating that lysosomal dysfunction is an early event in Cln6+/E200A− neuropathology. Cln6+/E200A− mice also prominently featured early detectable, widespread astroglisis and microglisis. It is unclear whether glial activation directly promotes neuronal cell loss, or presents a secondary, but easily detectable consequence of the disease process.

In summary, we described a girl with a de novo CLCN6 p.E200A variant, who presented a range of neurological defects that are suggestive of NCLs. The Cln6+/E200A− mouse model, the first one with a human disease-causing CIC-6 variant, demonstrates the importance of Cl−/H+ exchange activity for CIC-6 function. It phenocopies the CLCN6 p.E200A patients, and clinically and pathologically fulfills the characteristics of human NCLs. Therefore, our work establishes variants in CLCN6 as a novel genetic cause of NCLs via gain-of-function toxicity, highlighting the importance of considering CLCN6 mutations in the diagnosis of NCLs. Our study reveals a role of altered mTORC1-TFEB/TFE3 signaling and autophagy-lysosome function, the disturbance of lipid metabolism and glycometabolism, and apoptosis in CIC-
6-mediated NCLs. We also present the sequence of pathogenic events in neurodegenerating Clcn6<sup>E200A/V</sup> brains, with autophagic dysfunction, lysosome deficiency, and gliosis being early events leading to neuronal dysfunction. This is helpful for both understanding the molecular mechanism of NCLs and developing therapeutic treatments of related neurodegenerative disorders.

**Conclusion**

**Clcn6** variants interfere with the TFEB/TFE3 axis of mTORC1 signaling via a dominant effect, and cause NCLs. Moreover, Cl<sup>-</sup>/H<sup>+</sup>-exchange activity is critical to the physiological function of CIC-6.

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**Author Contributions**

H.H., T.S., and J.P. contributed to the conception and design of the study; all authors contributed to the acquisition and analysis of data; H.H., T.J.J., T.S., and J.P. contributed to drafting the text or preparing the figures. All authors approved the final version of the manuscript.

**Potential Conflicts of Interest**

Nothing to report.

**Data availability**

All data needed to evaluate the conclusions are present in the paper and/or the Supplementary Materials.

**References**


