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Macroautophagy is defective in mucolipin-1-deficient mouse neurons

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ABSTRACT

Mucolipidosis type IV is a neurodegenerative lysosomal disease clinically characterized by psychomotor retardation, visual impairment, and achlorhydria. In this study we report the development of a neuronal cell model generated from cerebrum of *Mcoln1*$^{-/-}$ embryos. Prior functional characterization of MLIV cells has been limited to fibroblast cultures gleaned from patients. The current availability of the mucolipin-1 knockout mouse model *Mcoln1*$^{-/-}$ allows the study of mucolipin-1-defective neurons, which is important since the disease is characterized by severe neurological impairment. Electron microscopy studies reveal significant membranous intracytoplasmic storage bodies, which correlate with the storage morphology observed in cerebral cortex of *Mcoln1*$^{-/-}$ P7 pups and E17 embryos. The *Mcoln1*$^{-/-}$ neuronal cultures show an increase in size of LysoTracker and Lamp1 positive vesicles. Using this neuronal model system, we show that macroautophagy is defective in mucolipin-1-deficient neurons and that LC3-II levels are significantly elevated. Treatment with rapamycin plus protease inhibitors did not increase levels of LC3-II in *Mcoln1*$^{-/-}$ neuronal cultures, indicating that the lack of mucolipin-1 affects LC3-II clearance. P62/SQSTM1 and ubiquitin levels were also increased in *Mcoln1*$^{-/-}$ neuronal cultures, suggesting an accumulation of protein aggregates and a defect in macroautophagy which could help explain the neurodegeneration observed in MLIV. This study describes, for the first time, a defect in macroautophagy in mucolipin-1-deficient neurons, which corroborates recent findings in MLIV fibroblasts and provides new insight into the neuronal pathogenesis of this disease.

Introduction

Mucolipidosis type IV (MLIV; OMIM 252650) is a progressive neurological lysosomal disease that usually presents during the first year of life with mental retardation, corneal opacities, elevated blood gastrin levels with achlorhydria, and delayed motor milestones (Berman et al., 1974; Frei et al., 1998). MLIV is an autosomal recessive disease with the majority of the cases (80%) reported in individuals of Ashkenazi Jewish (AJ) descent (Bargal et al., 2000). The carrier frequency in AJ is estimated to be 1:100 and the major AJ mutation, present on 72% of the AJ MLIV alleles, is an A–G transition at the 3′ acceptor site of intron 3 (Bargal et al., 2000; Bassi et al., 2000; Sun et al., 2000). The minor AJ mutation, found on 23% of the AJ MLIV alleles, is a 6434 bp genomic deletion that spans exons 1–6 and the first 12 bp of exon 7 (Goldin et al., 2004; Wang et al., 2002).

The *MCOLN1*-encoded protein (mucolipin-1, TRPML1) is a transmembrane protein with topological homology to other members of the TRP superfamily of ion channels. Several studies have begun to shed light on the function of mucolipin-1 as a calcium channel, however, the pathogenic mechanism by which loss of mucolipin-1 leads to cellular storage and neuronal cell dysfunction and death in MLIV is still poorly understood (Kiselyov et al., 2005; LaPlante et al., 2002, 2006; Manzoni et al., 2004; Miedel et al., 2006; Pryor et al., 2006; Thompson et al., 2007; Venkatachalam et al., 2006; Vergarajauregui and Puertollano, 2006).

Recently, an increased association between lysosomal diseases and macroautophagy dysfunction has been described. Macroautophagy is a constitutive, well orchestrated process responsible for bulk degradation of long-lived proteins and cellular organelles. Macroautophagy is essential for the survival of neural cells and its impairment is implicated in the pathogenesis of neurodegenerative disorders (Komatsu et al., 2006; Nedelsky et al., 2008; Ventruti and Cuervo, 2007). In some lysosomal diseases, the observed autophagic stress is also correlated with neuronal death and could be linked to the neurodegeneration, although the full extent of neuronal death in most lysosomal diseases has not yet been determined (Cao et al., 2006;

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Koike et al., 2005; Pacheco et al., 2007; Raben et al., 2009a,b; Settembre et al., 2008a,b).

We previously generated the first murine model for MLIV by targeted knockout of *Mcoln1*, and showed that the mouse model accurately replicates MLIV disease in humans. The *Mcoln1*$^{-/-}$ mice present with numerous dense inclusion bodies in all cell types in brain but particularly in neurons, severe retinal degeneration, elevated plasma gastrin, and vacuolization in parietal cells (Venugopal et al., 2007). More recently, a more detailed neuropathological characterization of the *Mcoln1*$^{-/-}$ mouse showed evidence of ganglioside accumulation throughout the CNS, presence of P62/SQSTM1 inclusions, reduced myelination in axonal tracts of the cerebrum and cerebellum and presence of axonal spheroids in white-matter tracts and Purkinje cell axons of the cerebellum (Micsenyi et al., 2009). We have recently shown that human MLIV fibroblasts have a defect in chaperone mediated autophagy (CMA) (Venugopal et al., 2009). Given the crosstalk between different autophagy pathways (Kaushik et al., 2008), herein we have investigated macroautophagy in a new neuronal culture model of MLIV. We show for the first time that macroautophagy is defective in *Mcoln1*$^{-/-}$ neurons, suggesting that defective autophagy contributes to the pathogenesis of neuronal loss in MLIV, the most devastating component of the human disease.

Materials and methods

Establishment of mouse neuronal cultures

Embryonic day 17 (E17) embryos resulting from heterozygous mating were dissected from the mother via cesarean section. Mouse neuronal primary cultures were established as previously described (Lesuisse and Martin, 2002; Reis et al., 2006). Briefly, both hemispheres of the cerebrum were dissected and treated with 0.5% trypsin, 0.1% trypsin inhibitor, and 228 U/ml DNAse I for 1 min each at 37 °C. The tissue was dissociated in neurobasal medium containing 2% B27 supplement and 1% penicillin/streptomycin by repeated passage through a P1000 and a P200 pipette. Cells were plated at a density of 2×10^6 cells/ml (~2000 cells/mm^2) and were cultured at 37 °C with 5% CO_2 and maintained in the same medium as above. Genotyping was done by PCR using genomic DNA from embryo tails as previously described (Venugopal et al., 2007).

Immunofluorescence and confocal microscopy

Staining with 50 nM LysoTracker (Invitrogen) was done in live cells for 15 min at 37 °C. Lamp1 staining was done with anti-lamp1 antibody clone 1DB4 (BD Biosciences) after fixation of neurons with 4% paraformaldehyde PFA (pH 7). Lamp1 antibody was diluted in 1% bovine serum albumin (BSA), 2% normal goat serum (NGS) and 0.05% saponin to a final concentration of 0.25 μg/ml. Secondary antibody used was a rat IgG conjugated with Alexafluor 488 (Molecular Probes) diluted in 1% BSA, 2% NGS and 0.05% saponin to a final concentration of 2 μg/ml. Coverslips were mounted with FluoromountG (Southern Biotech). Specimens were analyzed using a Leica SP5 confocal microscope. Quantification and measurement of LysoTracker-labeled vesicles was done using the MetaXpress cellular imaging analysis software (MDS Analytical Technologies), based on a single optical confocal plane per neuron. The chosen threshold of 2.1 μm, to define large vesicles, was determined based on visual comparison of wild type and knockout images, followed by measurement of vesicle width.

Electron microscopy on embryonic neuronal primary cultures

Analysis was performed at the Electron Microscopy facility at the Massachusetts General Hospital Pathology Service. Briefly, after removal of the culture medium, cells were fixed in Karnovsky's KII Solution containing 2.5% glutaraldehyde, 2.0% PFA, 0.025% calcium chloride in a 0.1 M sodium cacodylate buffer, pH 7.4 for 1 h. Cells were then resuspended in warm 2% agar. After solidification, the agar block was processed routinely for electron microscopy in a Leica Lynx™ automatic tissue processor. Specimens were post-fixed in osmium tetroxide, stained *en bloc* with uranyl acetate, dehydrated in graded ethanol solutions, infiltrated with propylene oxide/Epon mixtures, embedded in pure Epon, and polymerized overnight at 60 °C. For electron microscopy of the cerebral cortex tissues obtained from dissected pups or embryos, the same methodology was used with the exception of the 2% agar block step which was omitted and fixation was overnight. One-micron sections were cut, stained with toluidine blue, and examined by light microscopy. Thin sections were cut with an LKB 8801 ultramicrotome and diamond knife, stained with lead citrate, and examined in a Phillips 301 transmission electron microscope (Phillips Analytical). Images were captured with an AMT™ (Advanced Microscopy Techniques) digital CCD camera.

Western blot analysis

Cells were washed with PBS, harvested, lysed for 30 min at 4 °C in lysis buffer containing 50 mM $NaPO_4$, pH 7.4, 0.5% Triton X-100, 10% glycerol, type I protease inhibitor cocktail set (Calbiochem), 1 mM phenymethylsulfonyl fluoride and spun at 11,000*g* for 10 min. Lysates were resolved using 16% or 8% Tris–glycine gels (Invitrogen) prior to transfer to a polyvinylidene fluoride membrane (Bio-Rad). Detergent (1% NP-40) soluble and insoluble p62 fractions were isolated and analyzed as previously described (Vergarajauregui et al., 2008). Primary antibodies used were: rabbit polyclonal anti-autophagy APG86 (anti-LC3 AP 1802a) (1.25 μg/ml) (Abgent), mouse monoclonal anti-neuronal nuclei (NeuN) (1 μg/ml) (Millipore), rabbit polyclonal anti-p62/SQSTM1 (1:1000) (Biomol Int, LP), rabbit polyclonal anti-p70S6K (9 ng/ml), rabbit polyclonal anti-phospho-p70S6K (Thr389) (109 ng/ml), rabbit monoclonal anti-mTOR (30 ng/ml), rabbit polyclonal anti-phospho-mTOR (Ser2448) (136 ng/ml), rabbit polyclonal anti-ubiquitin antibody (68 ng/ml), rabbit polyclonal anti-beclin-1 (58 ng/ml) (Cell Signaling Technology). Anti-mouse or rabbit IgG secondary antibodies HRP-conjugated and peroxidase substrate were used following manufacturer's instructions (GE Healthcare). Bands were quantified using the Scanner/Software Quantity One 4.5.0 (Bio-Rad).

Statistical analysis

Results are expressed as the mean ± standard deviation throughout the text and figures and are representative of at least 3 independent experiments. Unpaired *t* test was performed using GraphPad Prism (GraphPad Software).

Results

Characterization of Mcoln1$^{-/-}$ *mouse neuronal cultures*

To further characterize the neuronal cell phenotype present in the MLIV mouse model, we established neuronal cultures from E17 cerebrum of *Mcoln1*$^{-/-}$ embryos and wild type littermates. It has been previously demonstrated that neuronal cultures established according to the protocol utilized in this study show expression of neuronal nuclei (NeuN), a neuronal marker, at day 10 of culture and do not show glial contamination until 20 days in culture. Therefore, to ensure neuronal purity in our experiments, cells were plated at a density of 2000 cells/mm^2 and in all experiments cells were cultured for a maximum of 12 days (Brewer et al., 1993; Lesuisse and Martin, 2002). NeuN expression was analyzed by Western blot and similar levels were found in *Mcoln1*$^{+/+}$ and *Mcoln1*$^{-/-}$ neuronal cultures (data not shown).