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Selectively increased sensitivity of cerebellar granule cells to AMPA receptor-mediated excitotoxicity in a mouse model of Batten disease

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Batten disease, a lysosomal storage disorder, is caused by mutations in the *CLN3* gene. The *Cln3*-knockout (*Cln3*$^{-/-}$) mouse model of the disease exhibits many characteristic pathological features of the human disorder. Here, we show that *Cln3*$^{-/-}$ mice, similarly to Batten disease patients, have a deficit in cerebellar motor coordination. To explore the possible cellular cause of this functional impairment, we compared the vulnerability of wild type (WT) and *Cln3*$^{-/-}$ cerebellar granule cell cultures to different toxic insults. We have found that cultured *Cln3*$^{-/-}$ cerebellar granule cells are selectively more vulnerable to AMPA-type glutamate receptor-mediated toxicity than their WT counterparts. This selective sensitivity was also observed in organotypic cerebellar slice cultures. Our results suggest that lack of the CLN3 protein has a significant influence on the function of AMPA receptors in cerebellar granule neurons, and that AMPA receptor dysregulation may be a major contributor to the cerebellar dysfunction in Batten disease.
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Introduction

Neuronal ceroid lipofuscinosis (NCL) is a group of recessively inherited lysosomal storage disorders characterized by progressive neurodegeneration (Goebel, 1995). So far eight genetically distinct forms of NCL have been identified (Cooper, 2003). All forms are characterized by the intracellular accumulation of autofluorescent ceroid- and lipofuscin-like lipopigment. Mutations in the *CLN3* gene are responsible for the development of the most prevalent, juvenile onset form of NCL, also called Batten disease (The International Batten Disease Consortium, 1995). The disease begins between 5 and 8 years of age, and the typical clinical symptoms are progressive vision loss, frequent occurrence of seizures, loss of motor skills and progressive cognitive decline, cumulatively leading to premature death in the late teens or early 20s, although some may live into their 30s. A deficit in motor coordination referred to as ataxia is one of the primary clinical features in Batten disease (Raininko et al., 1990). Cerebellar atrophy has been observed in patients with the disease using computer tomography and magnetic resonance imaging (MRI), and the severity of cerebellar atrophy correlated positively with disturbances in motor functions (Autti et al., 1996; Nardocci et al., 1995; Raininko et al., 1990). The *Cln3*-knockout (*Cln3*$^{-/-}$) mouse model of the disease (Mitchison et al., 1999) exhibits many characteristic pathological features of the human disorder including accumulation of autofluorescent lipopigments containing mitochondrial ATP synthase subunit c in neural tissue (Mitchison et al., 1999), selective loss of hippocampal GABAergic interneuron populations (Pontikis et al., 2004), widespread astrocytic and microglial activation (Pontikis et al., 2004), and retinal and optic nerve degeneration (Seigel et al., 2002; Sappington et al., 2003). Although, a general cerebellar atrophy has not been observed in *Cln3*$^{-/-}$ mice, dysfunction of the *Cln3*$^{-/-}$ mouse cerebellum was indicated by MRI analysis (Greene et al., 2001). Furthermore, microarray analysis of the *Cln3*$^{-/-}$ cerebellum revealed significant changes in the expression of many genes involved in diverse cellular processes (Brooks et al., 2003).

Here, we show that *Cln3*$^{-/-}$ mice, similarly to Batten disease patients, have a deficit in motor coordination. Dysfunction or death of cerebellar granule cells, the main excitatory (glutamatergic) cells in the cerebellum, has been found to be a major causative factor in cerebellar motor incoordination (Hashimoto et al., 1999; Jensen et al., 1999). Therefore, we tested several different signaling and cell death pathways in wild type (WT) and *Cln3*$^{-/-}$ cerebellar granule cell cultures by exposing them to different toxic insults. Since excitotoxicity has been proposed to be involved in the pathophysiology of Batten disease (Chattopadhyay et al., 2002), the effect of glutamate

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receptor overactivation was also examined. We have found that cerebellar granule cells cultured from *Cln3*$^{-/-}$ mice are selectively more vulnerable to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-type glutamate receptor overactivation than their WT counterparts, indicating an increased AMPA receptor function in the absence of CLN3. The selectively increased sensitivity to AMPA receptor-mediated toxicity was also observed in organotypic cerebellar slice cultures. Our results suggest that lack of the CLN3 protein has a significant influence on the function of AMPA receptors in cerebellar granule neurons, and that AMPA receptor dysregulation may be a major contributor to the cerebellar dysfunction and progressive neurological decline associated to Batten disease.

Materials and methods

Chemicals

Basal Eagle's medium (BME), neurobasal medium, minimal essential medium (MEM), heat-inactivated fetal calf and horse sera, B-27 serum replacement, glutamine, gentamicin sulphate, penicillin/streptomycin solution, Hank's balanced salt solution (HBSS), and Gey's balanced salt solution were purchased from Gibco BRL, Invitrogen Corporation (Grand Island, NY). The culture plates were the products of Corning (Corning, NY). AMPA, *N*-methyl-D-aspartate (NMDA), kainate, cyclothiazide, MK-801, GYKI 52466, SYM 2206, and glutamate were obtained from Tocris Cookson (Bristol, UK). Propidium iodide and Fluoro-Jade were from Molecular Probes (Eugene, OR) and Histochem Inc. (Jefferson, AR), respectively. All other chemicals, if not stated otherwise, were purchased from Sigma (St. Louis, MO).

Animals

In this study, 129S6/SvEv wild type (WT) and homozygous *Cln3*-knockout mice (*Cln3*$^{-/-}$; Mitchison et al., 1999) inbred on a 129S6/SvEv background were used. All procedures were carried out according to the guidelines of the Animal Welfare Act, NIH policies and the University of Rochester Animal Care and Use Committee.

Rotarod behavioral test

An accelerating rotarod (AccuScan Instruments, Inc., Columbus, OH) was used to measure motor coordination of 2-month-old *Cln3*$^{-/-}$ and WT mice. During the training period, mice were placed on the rotarod starting at 0 rpm and accelerating to 30 rpm over a period of 240 s. Animals were trained for three trials consisting of three consecutive runs. Trials were separated by a 30-min rest interval. Following training, animals rested for 4 h and then were tested for three test trials consisting of three consecutive runs with 30 min of rest between each test trail. The latency to fall during the testing period was calculated, and data were analyzed by unpaired *t* test with Welch's correction.

Cell cultures

Cerebellar granule cell cultures were prepared from 8-day-old WT and *Cln3*$^{-/-}$ mice as previously described (Kovács et al., 2004), with some modifications. Briefly, cerebella were dissected and meninges and blood vessels removed. Then cerebella were minced using a single edged razor blade with two passes at 90° to each other at approximately 0.5-mm intervals. Minced tissue was incubated in 0.25% trypsin solution at 37°C for 12 min, cells were dispersed by trituration in a DNase- and soybean trypsin inhibitor-containing solution (0.01% and 0.05%, respectively) and plated in 24-well culture plates (6×10^5 cells/well). The plates were precoated with 10 μg/ml poly-L-lysine (MW: 30 000–70 000) for 1 h. Cultures were maintained for 7 days at 37°C in a humidified atmosphere of 5% CO_2/95% air in Basal Eagle's medium (BME) supplemented with 10% heat-inactivated fetal calf serum, 20 mM KCl, 2 mM glutamine, and 50 μg/ml gentamicin (K25+S; BME contains 5 mM KCl). Cytosine-β-arabinofuranoside (5 μM) was added 24 h after plating to limit the number of non-neuronal cells to less than 5%.

Cortical cultures were prepared from 15- to 17-day-old WT and *Cln3*$^{-/-}$ mouse embryos as described previously (Kovács et al., 2001). Briefly, cerebral hemispheres were dissected, and meninges were removed. Cortices were carefully separated from striata and incubated at 37°C for 15 min in Hank's balanced salt solution containing 0.05% trypsin and 50 μg/ml gentamicin sulphate. Trypsinization was stopped by adding trypsin inhibitor and DNase to final concentrations of 0.05% and 0.01% respectively. After centrifugation with 180 *g* for 2 min, cortices were mechanically dissociated in culture medium by trituration with a 1-ml pipette tip attached to a 5-ml serological pipette. Cells were plated in 48-well plates (1.5×10^5 cells/well). The plates were precoated with 2 μg/ml poly-L-lysine (MW > 300 000) for 1 h. For dissociation, plating, and maintenance, Neurobasal medium supplemented with 2% B-27 serum replacement, 0.5 mM glutamine, and gentamicin sulphate (50 μg/ml) was used. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Half of the medium was changed on the 4th day in vitro (DIV 4) and then twice a week. Experiments were performed on DIV 6, 12–13, and 20.

Organotypic slice cultures

Cerebellar slices were prepared from 10-day-old WT and *Cln3*$^{-/-}$ mice as described by Marín-Teva et al. (2004). Briefly, cerebella were dissected out into cold Gey's balanced salt solution supplemented with 33.3 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin (GBSS-glucose-PS), and the meninges were removed. Parasagittal cerebellar slices (350 μm thick) were cut with a McIlwain tissue chopper and incubated in GBSS-glucose-PS at 4°C for 1 h. The slices were then placed on the membrane of Millipore 30-mm cell culture plate inserts (Millicell CM, Millipore, Bedford, MA) (3–5 slices per insert). The inserts with the tissue slices were transferred to 6-well plates, each well containing 1 ml of prewarmed culture medium (50% MEM, 25% HBSS, 25% heat-inactivated horse serum, 5 mM Tris–HCl, 27.8 mM glucose, 1 mM glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin). Slices were maintained at 36°C in an atmosphere of 5% CO_2/95% air. The medium was changed 1 h after putting the slices on the plate inserts then on the 3rd and 5th days in vitro.

Trophic factor deprivation and drug treatment

Trophic factor deprivation in cerebellar granule cell cultures was carried out on the 7th day in vitro. Cultures were washed twice either with K25-S (BME supplemented with 20 mM KCl, 2 mM glutamine, and 50 μg/ml gentamicin) or K5-S (BME supplemented with 2 mM glutamine and 50 μg/ml gentamicin), then the corresponding medium (K25-S or K5-S) was added to the cells for 24 h.

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