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Amlodipine prevents apoptotic cell death by correction of elevated intracellular calcium in a primary neuronal model of Batten disease (CLN3 disease)

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ABSTRACT

CLN3 disease (Spielmeyer-Vogt-Sjogren-Batten disease) is a severe pediatric neurodegenerative disorder for which there is currently no effective treatment. The disease is characterized by progressive neuronal death, which may be triggered by abnormal intracellular calcium levels leading to neuronal apoptosis. Previously, we demonstrated reversal of the calcium effect in a neuroblastoma cell line using amlodipine and other calcium channel antagonists. In the present studies, we developed a *CLN3* siRNA-inhibited primary rat neuron model to further study etoposide-induced calcium changes and apoptosis in CLN3 disease followed by recovery experiments with amlodipine. Our results show that intracellular calcium is significantly elevated in siRNA-inhibited cortical neurons after potassium chloride-induced depolarization. We were also able to show that amlodipine, a predominantly L-type dihydropyrimidine calcium channel antagonist can reverse the aberrant calcium elevations in this model of the disease. We performed an *in situ* TUNEL assay following etoposide-exposure to siRNA inhibited primary neurons, and apoptotic nuclei were detected providing additional evidence that increased neuronal apoptosis is associated with increased calcium levels. Amlodipine also reduced the absolute number of apoptotic cells in this experimental model.

1. Introduction

The neuronal ceroid-lipofusinoses (NCL's) are a group of recessively inherited neurodegenerative disorders. Clinically, they are characterized by progressive vision loss, seizures, motor and cognitive dysfunction and early death. Histopathologically, the disorders share similar features of massive neuronal cell death and the accumulation of a cytoplasmic autofluorescent storage material [1]. There are multiple clinical variants with fifteen identified genes to date [2–5]. CLN3 disease (Spielmeyer-Vogt-Sjogren-Batten disease, OMIM 204200) is caused by mutations in the *CLN3* gene on chromosome 16. It is classified as a lysosomal storage condition. Most children with this disease appear to develop normally until age 4–7 years when they present with progressive visual loss, seizures, and relentless motor and cognitive decline ultimately leading to death in the mid to late 20's.

Although the gene has been identified the mechanism through which the abnormal or missing protein leads to neuronal cell death is unclear [6,7]. The protein is a multispanning integral membrane protein which localizes to membrane lipid rafts and functions to insert a double bond into the palmitate molecule of palmitoylated membrane-associated proteins [8,9]. It has been shown by our group and others that downstream of this desaturase effect abnormal intracellular calcium accumulation may trigger apoptosis and that this process may be reversed by increased expression of the CLN3 protein [10–15]. It has been suggested that the calcium-induced cytotoxicity resulting from loss of CLN3 protein (CLN3P, Battenin) function may be mediated by the neuronal calcium sensor calsenilin [16]. Prior to this present study, we used an SH-SY5Y

Abbreviations: CLN, ceroid-lipofuscinosis neuronal (gene symbol); NCL, neuronal ceroid-lipofuscinoses; GFP, green fluorescent protein; siRNA, small inhibiting RNA.

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neuroblastoma cell model to screen known calcium-channel modulators for their ability to modulate the calcium elevations seen in CLN3 knock down SH-SY5Y cells. We identified amlodipine and several other predominantly L-type calcium channel antagonists as candidate drugs to lower intracellular calcium levels in this model [17]. Primary neurons are physiologically and morphologically more similar to *in situ* neurons than SH-SY5Y cells and represent a more accurate model of human disease pathophysiology. In this present study, we inhibited CLN3P in primary rat cortical neurons using siRNA and demonstrate that CLN3P-inhibited primary neurons have a significant elevation of intracellular calcium similar to that seen in SH-SY5Y cells. We were able to demonstrate that amlodipine at pharmacological levels can reverse the calcium elevation in primary neurons. We were also able to show that neuronal cell death in this model system involved apoptosis, a process that was reversed by the addition of amlodipine into the model system.

2. Materials and methods

2.1. Preparation of primary rat neurons

Embryonic rat cortical neurons were obtained from Sprague–Dawley rats (Dr. Marc Dichter, University of Pennsylvania), at 18 embryonic days (E18) and primary dissociated cultures were prepared from the embryonic hippocampi as described previously [18,19]. Briefly, hippocampi from E18 rat embryos were dissected from anesthetized pregnant Sprague–Dawley rats and trypsinized in Dulbecco's minimum essential medium (DMEM, Whittaker Bioproducts, Walkersville, MD) containing 0.027% trypsin at 4 °C for 20 min. They were then taken up into media consisting of DMEM supplemented with 10% bovine calf serum (Hyclone Laboratories, Logan, UT), 10% Hams F12 media with glutamine and 50 U/mL penicillin–streptomycin (Sigma Chemical Co, St Louis, MO). The neurons were plated on poly-L-lysine coated glass coverslips at a density 1×10^5 cells/cm^2 in serum-free Neurobasal medium (Gibco, Grand Island, NY) supplemented with B27 (Gibco) and incubated at 37 °C in 5% CO_2. On day 3 after plating, cells were infected with adenovirus at Multiplicity of Infection (MOI) = 25. Expression of green fluorescent protein (GFP) was detectable in cortical neurons by 72 h post-transduction and continued for the life of the culture. The highest rate of infection we obtained was 50–60%.

2.2. CLN3 silencing and adenoviral titer

CLN3 silencing was achieved using DNA vector-based siRNA technology as described previously [15]. Briefly, we designed two DNA sequences. The A sequence encoded a short hairpin RNA targeting the CLN3 gene: CTTGCCGAGTATTTCATTAA and a control sequence scrambled (scr) against siRNA-CLN3: GTGCCGTTCCTTATTATCTAA. In our earlier studies in neuroblastoma cells, a second siRNA probe was also used but was found to be less efficient than the one we have used in this study as it produced a significantly lower degree of knockdown [20]. The probes were cloned into a commercially available vector pRNAT-H1.1/Adeno (Genscript Corporation, Piscataway, NJ) with a coral GFP marker controlled by a cytomegalovirus (CMV) promoter for tracking transfection efficiency. The vector linearized with PmeI was cotransformed in BJ5183 *Escherichia coli* cells together with pAdEasy-1 Vector (Stratagene, CA). The positive recombinations were amplified in XL-gold competent cells. Plasmid was isolated using the HiSpeed plasmid midi kit (Qiagen, Valencia, CA) and transfected into a packaging cell line Ad293 (Stratagene, La Jolla, CA) to produce adenovirus. After phase 3 propagation, the high titer viral preparation was collected and centrifuged at 25,000*g* for 90 min at 4 °C. Titers were achieved at 1×10^9 green-forming units (Gfu)/ml by concentrating the virus. The adenoviral titer was determined using a flow cytometry method as described previously [21]. The viral concentration was determined from the average of the percentage of GFP-expressing cells in four serial experiments.

2.3. Calcium studies

A portion of the neurons were incubated in 1 μM amlodipine overnight prior to cytosolic calcium ($[Ca^{2+}]_i$) measurement. Neurons exhibiting GFP labeling were selected for perifusion studies. The perifusion procedure and $[Ca^{2+}]_i$ measurement have been described previously [22–24]. Briefly, the coverslips with attached neurons were incubated with 15 μM Fura-2 acetoxymethylester (Invitrogene/Molecular Probes, Eugene, OR) in Krebs–Ringer bicarbonate buffer (KRBB) (115 mmol/L NaCl, 24 mmol/L $NaHCO_3$, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 25 mmol/L HEPES, pH 7.4) with 5 mmol/L glucose and 0.25% bovine serum albumin for 35 min at 37 °C. Coverslips were then placed in a perifusion chamber and perifused with KRBB with 5 mmol/L glucose at a flow rate of 1 ml/min at 37 °C. After 5 min to reach a stable baseline, 15 mM KCl was applied for 5 min. Then this KCl stimulation was repeated. The $[Ca^{2+}]_i$ signal was measured with a dual wavelength fluorescence microscope using a Zeiss AxioVision system.

2.4. Observation of apoptosis in CLN3 inhibited primary neurons

The apoptotic cell death in the primary cortical neurons was studied after etoposide (20 μM) treatment for 1 h both in the presence and absence of amlodipine. Initially, cortical neurons were pretreated with different concentrations of amlodipine (0 μM, 0.5 μM, 1 μM, 10 μM, 20 μM) for 24 h prior to etoposide treatment. Etoposide-induced apoptosis was maximally suppressed in 1 μm amlodipine pretreated neurons for 24 h and higher concentrations resulted in a bell-shaped dose–response with less suppression at the higher levels (data not shown). Following this preliminary experiment, 1 μM amlodipine was selected for the further studies. Apoptosis was measured by a TUNEL study using the Apo-BrdU-Red *in situ* DNA fragmentation assay kit (Biovision, CA) according to the manufacturer's instructions. In brief, cultures were fixed in fresh 4% paraformaldehyde solution, and permeabilized with ethanol at 4 °C overnight. The cultures were incubated in the TUNEL reaction mixture for 1.5 h in the 37 °C incubator. Apoptotic nuclei were detected by TUNEL staining. Br-dUTP sites were identified by a red fluorescence labeled anti-BrdU monoclonal antibody. Total nuclei were labeled with diaminobenzidine (DAB). About 200 cells from three fields of vision in three separate experiments were counted using a Leica fluorescent microscope at a magnification of 20×. Three independent fields were counted in three separate experiments. Data were expressed as the percentage of TUNEL-positive cells in the total number of cells counted.

2.5. Western blot analysis

Western blots were used to determine the efficiency of CLN3 silencing at the protein level. Cells were trypsinized and collected after 72 h of siRNA treatment. Total protein was extracted from the cells using the Mammalian Protein Extraction Reagent (M-PER, Thermo Scientific, Rockford, IL). Twenty micrograms total protein of control, scrambled siRNA (scr) and CLN3 (−) siRNA was loaded in 4–15% SDS gels (Bio-Rad Laboratories, Hercules, CA). Protein was transferred from the gel to a PVDF membrane (BioRad, Hercules, CA) and the membrane was blocked overnight with 5% non-fat dry milk in Tris-Buffered Saline Tween-20 (TBST) (USB Corporation,