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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,

Cleveland, OH). After blocking, the membrane was washed three times with TBST and blotted overnight with anti-CLN3 antibody in 0.5% non-fat dry milk in TBST. After washing three times with TBST, the membrane was blotted with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (AbCam, Cambridge, MA) for 1 h at room temperature. The membrane was then washed three times with TBST. Protein bands were detected with ECL Plus (GE Healthcare, Piscataway, NJ) and the X-ray film scanned and band density calculated using the Quantity One software (BioRad, Hercules, CA). The CLN3 antibody was produced and purified as previously described [17,25]. It is a polyclonal antibody raised against the peptide sequence corresponding to amino acids 58–77 of the CLN3 protein and has been validated in a number of additional studies [26]. Total protein concentration in each cell lysate was measured using the Lowry method.

Data is given as mean and SD. *T*-tests were used to compare values. The significance level was set at $p < 0.05$.

3. Results

3.1. Efficiency of CLN3 silencing

The efficiency of silencing the CLN3 protein was determined by Western blot (Fig. 1A and B). Here it can be seen that siRNA treatment resulted in loss of the CLN3 protein.

3.2. Calcium studies in silenced primary neurons and effect of amlodipine

Potassium chloride-induced depolarization significantly increased intracellular calcium in CLN3 protein inhibited primary neurons when compared to control cortical neurons in a manner consistent with our earlier studies with neuroblastoma cells [17]. Incubation with amlodipine normalized the potassium chloride-induced elevation of calcium in CLN3 protein inhibited cells to baseline (Fig. 2).

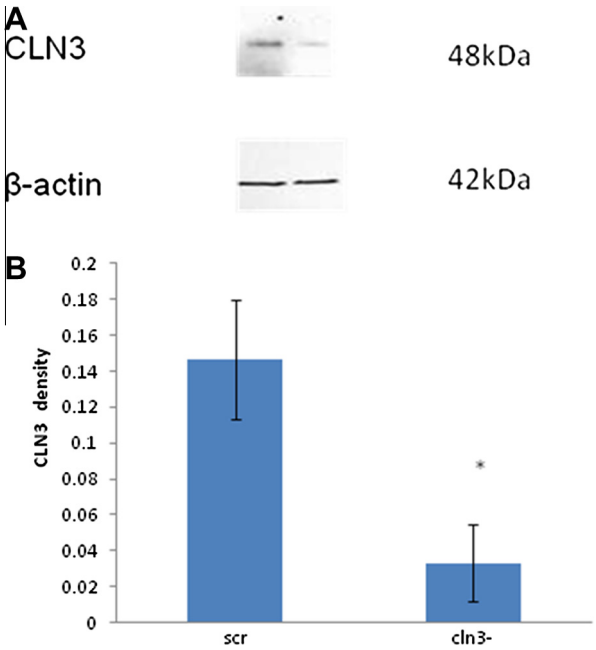

Fig. 1. The efficiency of silencing CLN3 was determined by Western blot analysis of the CLN3 protein. β-Actin was used as control protein. (A) Lane 1 scr RNA, Lane 2 CLN3 (−) siRNA. (B) Densitometric scanning of Western blot normalized to the β-actin. Data is presented as mean ± standard deviation ($n = 3$), $^*p < 0.05$.

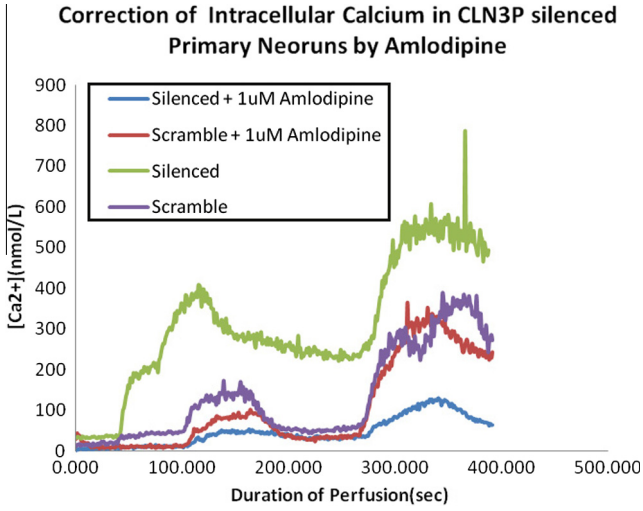

Fig. 2. Comparison of intracellular calcium at each time point during perifusion. Duration of perifusion is in seconds with each time point (1–500) representing 5 s, total time 33 min. The experiment was repeated three times with consistent results. Data averaged per time point over at least 3 neurons per category.

Our study also demonstrated that the potassium chloride-induced calcium elevation observed over 120 s was persistently maintained in the CLN3 protein inhibited cells when compared to control cortical neurons. Amlodipine significantly decreased the elevation of calcium in CLN3 protein inhibited cells and normalized it to that of the concentrations we observed in control cortical neurons (Fig. 3, $p = 0.03$). This elevation of total calcium concentration suggests that knocking down the CLN3 protein in primary cortical neurons results in abnormal calcium channeling.

3.3. Studies of neuronal apoptosis

We used the TUNEL study to primarily determine the effect of amlodipine treatment on apoptotic cell death induced by exposure to etoposide (Fig. 4). The primary cortical neurons showed increased apoptotic cell death in CLN3 knock down cells when com-

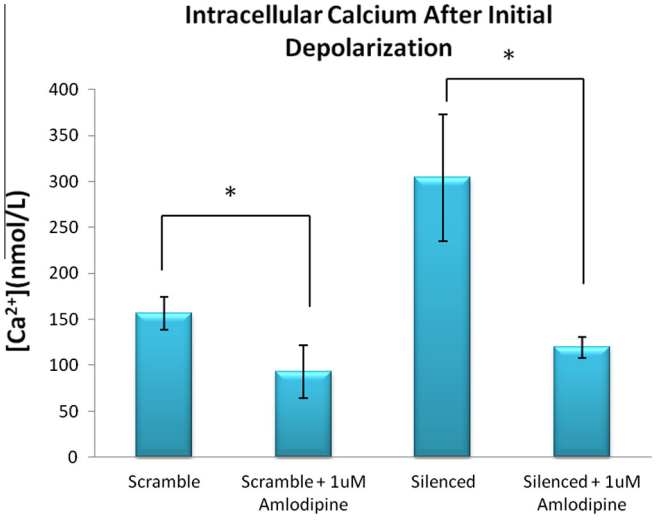

Fig. 3. Sum of calcium levels during 120 time points (600 s) following initial KCl-induced depolarization peak, averaged over at least 3 neurons. Intracellular calcium in CLN3P-inhibited primary neurons is elevated compared to controls. Amlodipine treatment decreases intracellular calcium in both control primary neurons and CLN3P-inhibited cells.

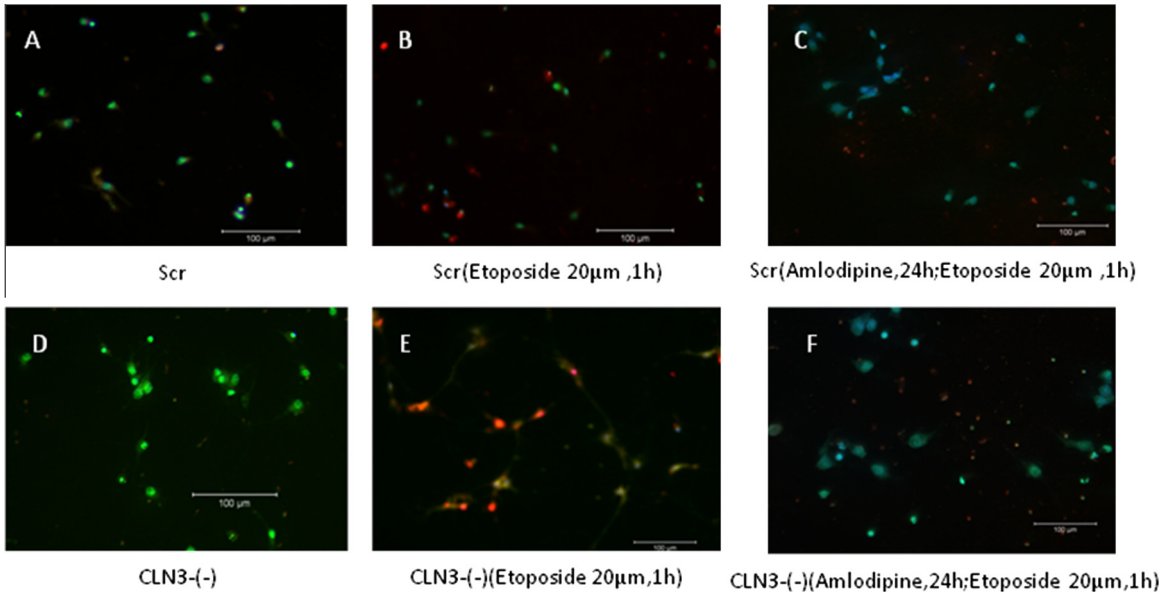

Fig. 4. Cell survival with amlodipine (A). Total nuclei were labeled with diaminobenzidine (DAB). Br-dUTP sites are labeled with Texas red. The effect of etoposide (Etop)-induced apoptosis in primary cortical neurons determined by TUNEL staining. The apoptotic cell death in neurons was studied after etoposide (20 μM) treatment for 1 h both in the presence and absence of amlodipine (1 μM). (A) scr; (B) scr treated with Etop; (C) scr treated with A and Etop; (D) CLN3-(−) treated with Etop; (E) CLN3-(−) treated with Etop; (F) CLN3-(−) treated with A and Etop. Scale bar: 10 μm.

pared to the siRNA scrambled control cells (Fig. 4B and E). Cell death by apoptosis decreased in both groups after treatment with amlodipine (Fig. 4C and F). Amlodipine pretreatment decreased TUNEL positive cells from 62% ± 12.12 to 48% ± 13.32 ($p = 0.4$) in scr cells and from 89% ± 2.31 to 54% ± 5.51 ($p = 0.05$) in CLN3-silenced cells. These results demonstrate that CLN3-silenced primary neurons exhibit a greater degree of etoposide-induced apoptosis than control cells and that treatment with amlodipine protects from etoposide-induced apoptosis in all neurons but with a larger and significant effect in the siRNA-silenced cortical neurons.

4. Discussion

The purpose of our ongoing research is to investigate and develop treatment strategies that prevent neuronal apoptosis and neurodegeneration in CLN3 disease. It is our hypothesis, and that of others [27,28] that the neuronal death and subsequent massive neuronal loss seen in CLN3 disease is due to neuronal apoptosis or impaired autophagy. In addition, apoptosis is triggered by pathologically elevated intracellular calcium levels [17]. To study this process we established a model using CLN3 silenced primary cortical neurons to measure calcium changes in CLN3 disease. Using our model we demonstrated that intracellular calcium levels are elevated in primary neuronal cells lacking CLN3 protein.

We were also able to show that the increased intracellular levels of calcium in the CLN3 protein inhibited neurons was associated with an increased rate of etoposide-induced apoptosis based on TUNEL staining, consistent with our hypothesis of the pathway of neuronal death in CLN3 disease. This particular study did not evaluate the potential for impaired autophagy as a second mechanism but that process remains a possible additional mechanism for the neuronal death [28].

Finally, we also show that amlodipine, a drug previously screened to have an effect of decreasing the elevated intracellular calcium levels in *CLN3* siRNA knock down in SH-SY5Y neuroblastoma cells, is also capable of decreasing the intracellular calcium levels in CLN3 protein-inhibited primary cortical neurons [17]. The importance of this present study using primary neurons relates to the resilience to induced death in the neuroblastoma cells which we used previously in which we could not demonstrate apoptosis in association with the increased cellular calcium. In other studies, amlodipine was shown to inhibit excessive neuronal apoptosis in cerebellar granule cells and was also demonstrated to prevent cytotoxicity in cortical neurons isolated from hypertensive rats [29,30]. In our experiments, the amlodipine effect was seen not only in the CLN3 knocked down primary neurons but also to a lesser and non-significant effect in the control experiments consistent with these observations of a non-specific "neuroprotective" effect of amlodipine [31]. These and our own studies indicate that agents such as amlodipine that primarily block the L-type voltage Ca^{2+} channels and also have effects on N-type and P/Q type calcium channels [32,33] have distinct neuroprotective properties that indicate they have the potential to be used as a treatment of other causes of neuronal death in addition to CLN3 disease. At least one additional potential benefit of a drug such as amlodipine is that it has been shown to reduce the effects of oxidative stress in a number of tissues when it is used in its approved fashion to control hypertension [34–36]. Others have shown in a fruit fly model that defective CLN3 results in impaired response to oxidative stress [37] providing additional compelling evidence to suggest value in the use of this type of drug. The effects of amlodipine on multiple calcium channel types might also explain the biphasic effect that we observed in our dose titration experiment as multiple calcium channels are present in brain cells.

Although the function of the CLN3 protein is debated, its absence has been convincingly shown to play a role in the induction of neuronal death [15]. Our studies indicate that the downstream effect of CLN3 protein deficiency or loss of function impacts intracellular calcium levels. It is known that the increased levels of calcium trigger apoptotic pathways and lead to neuronal cell death. Modification of this apoptotic signaling provides us with a potential rational therapeutic target for CLN3 disease. Our studies indicate that amlodipine, which is a dihydropyrimidine calcium antagonist that has already been approved by the FDA for other medical indications including high blood pressure and angina pec-

toris, is a suitable modulator of these changes *in vitro*. Amlodipine is known to cross the blood–brain barrier in a facilitated manner that may not be as efficient as the transport of other dihydropyridine calcium channel antagonists such as nifedipine [38] and it seems appropriate to hypothesize that any potential therapies for this neurodegenerative disease need to reach neurons. Further studies need to be done to determine if normalizing the siRNA-induced increases in intracellular calcium levels can also prevent neuronal death in CLN3 disease. This question may be best answered using an animal model and probably evaluating a range of FDA-approved dihydropyridines that are known to be transported into the brain.

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