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Screening for calcium channel modulators in CLN3 siRNA knock down SH-SY5Y neuroblastoma cells reveals a significant decrease of intracellular calcium levels by selected L-type calcium channel blockers

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Abstract

Background—Defects of the CLN3 gene on chromosome 16p12.1 lead to the juvenile form of neuronal ceroid-lipofuscinosis (JNCL, Batten Disease), the most common recessive inherited neurodegenerative disorder in children. Dysregulation of intracellular calcium homeostasis in the absence of a functional CLN3 protein (CLN3P, Battenin) has been linked to synaptic dysfunction and accelerated apoptosis in vulnerable neuronal cells. Prolonged increase of intracellular calcium concentration is considered to be a significant trigger for neuronal apoptosis and cellular loss in JNCL.

Methods—We examined the potential effect of 41 different calcium channel modulators on intracellular calcium concentration in CLN3 siRNA knock down SH-SY5Y neuroblastoma cells.

Results—Six drugs belonging to the group of voltage dependent L-type channel blockers show significant lowering of the increased intracellular calcium levels in CLN3 siRNA knock down cells.

Conclusions—Our studies provide important new data suggesting possible beneficial effects of the tested drugs on calcium flux regulated pathways in neuronal cell death. Therapeutic intervention in this untreatable disease will likely require drugs that cross the blood-brain barrier as did all of the positively screened drugs in this study.

General Significance—Better comprehension of the mechanism of neurodegeneration in rare recessive disorders, such as neuronal ceroid-lipofuscinoses, is likely to help to better understand mechanisms involved in more complex genetic neurodegenerative conditions, such as those associated with aging.

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Keywords

Juvenile neuronal ceroid-lipofuscinosis; CLN3 protein; intracellular trafficking; L-type calcium channels; palmitoyl-protein Δ -9 desaturase

1. Introduction

Defects of the CLN3 gene on chromosome 16p12.1 lead to the juvenile form of neuronal ceroid-lipofuscinosis (JNCL, Batten Disease). As a group, the neuronal ceroid-lipofuscinoses represent the most common recessive inherited neurodegenerative disorders in children, with an overall incidence ranging from 1/12500 to 1/21000 [1]. At least ten different clinical forms and nine gene defects have been identified so far [2]. Due to rapidly progressive retinal degeneration, JNCL patients develop visual impairment between 4 and 7 years of age, leading ultimately to blindness. Relentless neurological degeneration accounts for brain atrophy, abnormalities of grey and white brain matter, and leads clinically to severe motor and cognitive handicap. Histopathological analysis by electron microscopy shows a typical fingerprint inclusion storage pattern of the accumulated autofluorescent storage material lipofuscin and the brain shows massive neuronal loss [3]. Mutation analysis is available for confirmation of a clinical diagnosis since the gene was cloned in 1995 [4]. There exists a therapeutically unmet need in this disorder, as the existing treatment is only symptomatic and cannot prevent the relentless deterioration and early death of affected patients.

The CLN3 protein (Battenin) is a hydrophobic transmembrane protein, which has been shown to localize to lipid rafts [5, 6]. It is involved in intracellular trafficking and regulation in neuronal and non neuronal cells [1, 7, 8]. CLN3P has been found to have an anti-apoptotic effect in cultured cells and it is implicated in several important signaling pathways [9]. Defects in many of the signaling pathway proteins interacting with CLN3P lead to apoptotic cell death, therefore underlining the possible modulating functions of CLN3P as an anti-apoptosis protein [1,10,11]. A strong interplay between CLN3 and the calcium dependent multifunctional protein calsenilin has been shown recently in CLN3 knock down and overexpressing SH-SY5Y cells, as well as in brain tissues of CLN3 knock out mice. Increased calcium concentrations in vitro and in cells decreased the affiliation between CLN3 and calsenilin in a concentration-dependent manner. Calsenilin expression was reduced and calcium mediated apoptosis was prevented in CLN3 overexpressing SH-SY5Y cells [12].

Apoptotic cell death in neuronal cells is thought to be triggered by different stimuli. Several lines of evidence support a close relationship between excessive intracellular calcium concentrations, rise of mitochondrial matrix free calcium and neuronal injury. Intracellular calcium overload is thought to activate calcium dependent processes in a common final network of different pathways leading to apoptotic neuronal cell death [13, 14, 15]. It has been previously shown in murine CLN3 $-/-$ cortical neurons [16] and in CLN3 knock down SH-SY5Y cells [12] that intracellular calcium homeostasis is disturbed in the absence of CLN3P.

The present study was aimed at testing the effect of 41 known calcium channel modulators on intracellular calcium concentrations in CLN3 siRNA knock down SH-SY5Y neuroblastoma cells [17]. The results show that six drugs belonging to the group of voltage dependent L-type (L-/T-type) calcium channel modulators, and are all known to cross the blood-brain barrier, show significant lowering of intracellular calcium levels in CLN3 siRNA knock down cells.

2. Materials and Methods

2.1. Cell culture

An SH-SY5Y cell line derived from a human neuroblastoma cell line was purchased from ATCC (Manassas, VA). SH-SY5Y cells were grown at 37°C and 5% CO₂ in DMEM (Gibco, MD) supplemented with 10% FBS in T-75 culture flasks until they reached 80% confluence. The cells were detached from the flask with trypsin 0.25% (Gibco, MD) and seeded in 96 well optical plates (Nalge Nunc International, NY), each well containing 125,000 cells. The cells attached to the plate within 24 hours, allowing infection with adenoviral particles (Stratagene, TX) containing either scrambled or silenced CLN3 vector sequences, controlled by a U6 promoter sequence (GenScript Corp., NJ). During 72 hours the virus was allowed to replicate and express siRNA to inhibit CLN3P expression. The cells were then used for calcium measurements.

2.2. Small interfering RNA of CLN3 to knock down CLN3P function

We knocked down CLN3P by using our previously described DNA vector-based siRNA technology [17]. In short, we cloned small DNA inserts encoding short hairpin RNA (siCLN3: CTTTGCCGAGTATTTTCATTAA at position 1012; siCLN3-scramble: GTGCCGTTCTTATTATCTAA, sequence scrambled from the siRNA sequence) and targeting the CLN3 gene into a commercially available vector (SD 1219: pRNAT-H1.1/Adeno; Genscript Corporation, Piscataway, NJ). The vector has a coral green fluorescent protein (cGFP) expression cassette to track the transfection efficiency. The AdEasy Adenoviral Vector system (Stratagene, La Jolla, CA, USA) was used to generate recombinant adenovirus. According to the previously described standard protocol [18], recombinant replication-deficient adenoviruses expressing GFP were generated and used to co-transfect the siRNA vector. Plasmid preparations were obtained from XL-10 gold competent cells. Plasmids were transfected into AD293 cells by using the Lipofectamine protocol (Invitrogen, La Jolla, CA). The transfection efficiency was followed by cGFP expression and its target was set at greater than 95%. The virus was harvested after 1 week from the AD293 cells and used for subsequent transduction to SH-SY5Y cells.

2.3. Antibody

CLN3P antibody was produced as previously described [19]. We raised these antisera against the peptide fragment corresponding to amino acids 64–76 of CLN3P. The antiserum was purified using Sulfolink columns according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). The fractions containing protein were dialysed overnight at 4°C in phosphate-buffered saline. The specificity of the antibody was tested by neutralizing it with the peptide sequence against which the antibody was raised.

2.4. Western Blot Analysis

The CLN3 antibody used in this study is a polyclonal antibody raised against the peptide sequence corresponding to amino acids 58–77 of the CLN3 protein. Total cellular extracts for CLN3 detection were prepared from SH-SY5Y cells transfected with siCLN3 or siCLN3-scramble. Cells were washed with cold PBS and lysed on ice in M-PER (Thermo Scientific, IL). The lysate was collected and clarified by centrifugation at 12,000g for 10 min at 4°C. Total protein was measured in the supernatant by the Lowry method. Electrophoresis of each sample containing 20–30 µg of total protein was performed on a 4–20% LongLife Gel (NuSep, Invitrogen, CA) in Tris Hepes Running Buffer (NuSep, Invitrogen, CA). The gel was transferred onto a blotting membrane and blocked by incubation in 0.5% non fat milk in TBST buffer (Millipore, MA) for 1 h at 25°C, followed by incubation with CLN3 antibody (IgG fraction) diluted in TBST buffer for 30 min at

25°C. After extensive washes with TBST buffer, the membrane was incubated in a 1:1000 dilution of anti-rabbit antibody IgG conjugated with horseradish peroxidase for 30 min at 25°C. The blot was washed and developed using the chemiluminescence detection system (VersaDoc) (Figure 1).

2.4. Calcium measurements

Cells were loaded with 10 μ M of the free calcium indicator Fura-2AM (Sigma-Aldrich, MO) and with 1 μ M of each of the 41 calcium modulating drugs (Sigma-Aldrich, MO) in HEPES Buffer. They were incubated for 30 minutes before intracellular calcium levels were measured in a Flexstation microplate reader (Molecular Devices, CA). The SH-SY5Y cells were stimulated at 30 and at 100 seconds with 10 mM potassium chloride delivered through the Flexstation's pipetting system. The intracellular calcium was monitored for 30 seconds prior to stimulation and for a total duration of 230 seconds. As a second and third step, 1% TritonX100 and 50 mM EGTA were added successively. The Fura-2-fluorescence was measured at excitation wavelengths of 340 and 380 nm, and emission at 505 nm.

2.5. Calculation of intracellular calcium

The intracellular calcium concentrations were calculated from the 340/380 nm ratio by using the equation previously described [20]. Baseline calculations were obtained by averaging measures of calcium concentrations in 29 second time intervals before the KCl stimulation at 30 seconds and 100 seconds, as well as during the cells' recovery time at 140 seconds. The values of the increased calcium after both depolarization points at 30 seconds and 100 seconds were obtained by subtraction of the baseline value from the peak value. Adding the baseline value to the peak allowed for estimation of overall intracellular calcium concentrations. We examined values in siRNA knock down cells as well as in controls. The effect of 41 calcium channel modulators on intracellular calcium levels was compared to untreated cells.

2.6. Statistical analysis

Mean calcium concentrations, as well as their standard deviations ($\pm$ SD) were established. Standard statistical analysis was performed using the Student's paired one-tail t-test. P-values < 0.05 were considered significant.

3. Results

3.1. Baseline changes of intracellular calcium concentration after KCl stimulation

3.1.1. Calcium baselines before and after KCl stimulation in untreated cells—

Prior to KCl induced cell stimulation, the baseline intracellular calcium concentration was found to be about 10 nM higher in SH-SY5Y siRNA knock down cells than in siRNA scrambled control cells. After the first KCl induced cell stimulation at 30 seconds it shifted upwards by about 130% in both cell populations. After the second KCl induced cell stimulation, both the siRNA knock down cells, as well as the control cells maintained their secondarily elevated calcium concentration and did not reach the initial lowest calcium baseline. These changes were not found to be statistically significant ($p > 0.05$) (Table 1).

3.1.2. Calcium baselines before and after KCl stimulation in cells treated with L-type calcium channel modulators—

When incubating SH-SY5Y siRNA knock down and scramble control cells with L-type calcium channel modulators Amlodipine, Flunarizine, R(+) Bay K8644, Nimodipine, Nicardipine and Nifedipine, baseline intracellular calcium concentrations were lowered at all three time points. Treated siRNA knock down cells showed significantly lower baseline intracellular calcium concentrations

(20–50 nM) when compared to untreated siRNA knock down control cells after KCl induced cell depolarization at 30 ($p<0.05$) and at 100 seconds ($p<0.005$) (Table 2, Figure 2).

3.2. KCl induced intracellular calcium peak concentration

3.2.1. KCl induced intracellular calcium peak concentration in untreated cells

—Untreated SH-SY5Y siRNA scramble control, as well as siRNA knock down cells showed comparable increases over the intracellular baseline calcium concentration after KCl stimulation at 30 and 100 seconds. There was no significant difference ($p>0.05$) between the two cell populations (Table 3).

3.2.2. KCl induced intracellular calcium peak concentration in treated cells—

After incubation of SH-SY5Y cells with calcium channel modulators Amlodipine, R(+) Bay K8644, Flunarizine, as well as Nimodipine, Nicardipine and Nifedipine, intracellular calcium concentrations were found to be significantly lower in both siRNA scramble control and siRNA knock down cells ($p<0.05$). The intracellular calcium concentrations in control cells were lowered by about 10 nM, as compared to a decrease of about 20 nM in treated siRNA knock down cells after both depolarization time points. A statistically significant ($p<0.05$) lower intracellular calcium peak concentration was found in the siRNA knock down cells after depolarization at 100 seconds (Table 4, Figure 3A). Interestingly, incubating siRNA SH-SY5Y cells with calcium channel modulators S(–) Bay K8644 and (+)Verapamil led to elevation of calcium peaks as a response to the KCl induced cell depolarization, when compared to their untreated controls. Pre-incubating siRNA knock down cells with S(–) Bay K8644 and (+)Verapamil lead to a significant ($p<0.05$) increase of the calcium concentrations of about 30 nM after depolarization at 30 and 100 seconds (Table 5, Figure 3B).

3.3. Total KCl induced changes of intracellular calcium concentration

When adding the values obtained after KCl depolarization for intracellular calcium peak and baseline measurements, the resulting calcium concentrations in treated SH-SY5Y siRNA knock down and siRNA scramble control cells are as follows.

In SH-SY5Y siRNA scramble control cells, incubation with calcium channel modulators Amlodipine, R(+) Bay K8644, Flunarizine, as well as Nimodipine, Nicardipine and Nifedipine resulted in a 30 nM lower total intracellular calcium concentration after both KCl induced cell depolarizations at 30 and 100 seconds. Treated siRNA knock down cells were found to show a statistically significant ($p<0.005$) decreased intracellular calcium level of about 50 nM, when compared to the untreated cell population after both cell depolarizations (Table 6).

3.4. Baseline and intracellular calcium peak concentrations in cells after incubation with various calcium channel type modulators

Table 7 enumerates thirty two various types of calcium channel modulators. Incubating SH-SY5Y siRNA scramble control and siRNA knock down cells with these drugs did not show any effect on baseline or intracellular calcium peak concentration.

4. Discussion

In our present study we focused on identifying an intracellular calcium modulator that was capable of decreasing elevated intracellular calcium levels in the absence of CLN3P. We hypothesized that intracellular calcium changes may indicate altered membrane functionality in JNCL. Due to readily available techniques to study calcium changes, we therefore screened 41 calcium channel modulators and their effect on intracellular calcium levels in

CLN3 siRNA knock down SH-SY5Y neuroblastoma cells. Baseline calcium concentrations and the response to KCl induced depolarization in the CLN3 siRNA knock down cells were indistinguishable from the CLN3 siRNA scramble cells. Similar findings in primary mouse neurons were previously explained by the ability of primary neurons to form functional synapses in culture [16]. Even though SH-SY5Y neuroblastoma cells are undifferentiated neuronal cancer cells, they do show similar behavior to neuronal cells such as neurite growth, forming of functional synapses and expressing a limited number of receptors. Pre-incubating our SH-SY5Y neuroblastoma cells with selected L-type calcium channel antagonists Amlodipine, R(+) Bay K8644, Flunarizine, as well as Nimodipine, Nicardipine and Nifedipine led to significant decrease of baseline intracellular calcium concentrations, of KCl induced calcium peaks, as well as of the summarized (baseline + peak) intracellular calcium concentrations at both stimulation points in CLN3 siRNA knock down cells (tables 2, 4, 6). Pre-incubating CLN3 siRNA knock down cells with S(-) Bay K 8644 and (+) Verapamil led to significant increases in the calcium peaks in response to the KCl induced cell depolarization at 30 and 100 seconds (table 5). Molecule specific action in our SH-SY5Y cells probably accounts for these effects. In our experiment only certain L-type calcium channel modulators showed a significant effect on CLN3 siRNA knock down cells. The involvement of voltage gated L-type calcium channels in the complex signaling network regulating excessive elevation of intracellular calcium levels in the absence of a functional CLN3P is probably only partial. As SH-SY5Y neuroblastoma cells do not show complete similarity to neuron-like properties, we need to confirm the drug effect in primary neuronal cells. Interestingly, a recent study in CLN3 $-/-$ primary mouse neurons showed a prolonged recovery from depolarization when blocking N-type calcium channels with ω -conotoxin GVIA, but not when both N- and L-type channels were blocked. In the absence of CLN3P, negative regulation of N-type voltage gated calcium channels by an upregulated G protein β 1 subunit, after forming a stable complex with the γ subunit, was found to be causative [16]. Showing significant lowering of calcium levels by incubating our CLN3 siRNA knock down neuroblastoma cells with selected L-type calcium channel antagonists may indicate a G protein independent effect. Our study yielded comparable results, primarily involving L-type voltage gated calcium channels, with KCl depolarized CLN3 siRNA knock down SH-SY5Y cells.

Intracellular calcium overload is likely to be a part in the complex mechanism which triggers distinct signaling pathways and mediates neuronal cell death in infantile, late infantile and juvenile forms of neuronal ceroid-lipofuscinoses [12, 21]. L-type voltage gated calcium channels in lipid rafts are known to selectively increase neurotransmitter release and synaptic transmission via calcium signaling [15]. Changes in the regulation of neurotransmitter release, neurotransmission, neuronal cell structure and development have been described as mechanisms by which the deficient CLN3P contributes to neurodegeneration in Batten disease [16]. CLN3P accounts for a galactosylceramide binding domain which facilitates trafficking to lipid rafts through recycling endosomes [22, 23]. The abnormal CLN3P is known to be retained within the Golgi, failing to reach the lipid rafts [24]. This is thought to create a stress to the endoplasmic reticulum, leading to inappropriate calcium response and elevated mitochondrial membrane permeability [12]. It is likely that the localization of the transmembranous CLN3P to lipid rafts [6] makes its interaction with calcium homeostasis balancing mechanisms via L-type calcium channels possible. Influencing calcium transients through calcium channel modulators probably changes the biochemical environment in which CLN3P functions as a palmitoyl-protein Δ -9 desaturase [17]. Palmitoylation of proteins is known to regulate receptor clustering and stability in neuronal cells [25, 26]. CLN3P is hypothesized to be capable of acting upon multiple lipid-raft-resident proteins, many of which are translocated into membranes by palmitoylation [1, 17]. Failure to insert a double bond in the palmitate moiety of palmitoylated membrane proteins resulting from impaired or absent desaturase activity is likely to lead to alteration of

the physical state of the protein's fatty acid side-chain. Consequently, this may impact the protein's signaling or channeling function, and lead to disruption of the lipid raft [1]. Dysfunction of CLN3P may be causative to dysruption of calcium mediated pathways, either by being retained in the Golgi or by being absent from the plasma membrane.

In summary, our study has demonstrated that selected L-type calcium channel blockers including Amlodipine, R(+) Bay K8644, Nimodipine, Nicardipine, Nifedipine as well as the L-/T-type channel blocker Flunarizine show significant lowering of intracellular calcium levels in CLN3 siRNA knock down SH-SY5Y neuroblastoma cells. As increase in intracellular calcium is considered to be a significant trigger for neuronal apoptosis and cellular loss in JNCL, our studies provide important new data suggesting possible beneficial effects on calcium flux regulated pathways in neuronal cell death. Further studies need to be done in primary neuronal cells to confirm the potential drug effect as the next stage in developing treatment regimens. Therapeutic intervention in this untreatable disease will likely require a poly-drug therapy with molecules that cross the blood-brain barrier as did all of the positively screened drugs in this study.

Abbreviations

CLN3	Ceroid lipofuscinosis type 3
JNCL	Juvenile neuronal ceroid-lipofuscinosis
CLN3P	CLN3 protein
siRNA	small interfering ribonucleic acid
INCL	Infantile neuronal ceroid-lipofuscinosis

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Figure 1. CLN3 Western Blot demonstrating knock down of CLN3 by siRNA in SH-SY5Y cells
Western Blot analysis was performed as described in Materials and Methods. Expression of CLN3P is shown in siRNA knock down SH-SY5Y cells (lanes 1 and 2), siRNA scramble control SH-SY5Y cells (lane 3) and wild type SH-SY5Y cells (lane 4). Expression of β -actin is shown in siRNA knock down SH-SY5Y cells (lane A), siRNA scramble control SH-SY5Y cells (lane B) and wild type SH-SY5Y cells (lane C).

Figure 2. Results of intracellular calcium baselines [nM/mg protein] before and after KCl depolarization in cells treated with L-type calcium channel modulators

Baselines [nM/mg protein] in cells treated with Amlodipine, Flunarizine, R(+) Bay K8644, Nimodipine, Nicardipine and Nifedipine versus untreated cells (n=28). Treated siRNA knock down cells showed statistically significant lowering of the intracellular calcium baseline values during the cells' recovery time after KCl depolarization at 30 seconds ($p<0.05$) and at 100 seconds ($p<0.005$).

A **B**

Figure 3. Results of intracellular calcium peak concentrations [nM/mg protein] after KCl depolarization in cells treated with L-type calcium channel modulators

(A) Calcium peak concentrations [nM/mg protein] in cells treated with Amlodipine, Flunarizine, R(+) Bay K8644, Nimodipine, Nicardipine and Nifedipine versus untreated cells (n=28). Treated siRNA knock down cells showed statistically significant lowering of the intracellular calcium peak concentrations after KCl depolarization at 100 seconds ($p < 0.05$).

(B) Calcium peak concentrations [nM/mg protein] in cells treated with S(-) Bay K8644 and (+) Verapamil versus untreated cells (n=8). Treated siRNA knock down cells showed

statistically significant increase of the intracellular peak calcium concentrations after KCl depolarization at 100 seconds ($p<0.05$).

Table 1

Mean ($\pm$ SD) intracellular calcium baselines [nM/mg protein] before and after KCl depolarization in untreated cells (n=68).

Time Intervall	0–29 sec	70–99 sec	140–169 sec
Celltype			
Control cells	104 ($\pm$ 64)	131 ($\pm$ 79)	135 ($\pm$ 68)
siRNA cells	114 ($\pm$ 60)	141 ($\pm$ 68)	144 ($\pm$ 66)
One-tail p	0.14	0.27	0.17

Table 2

Mean ($\pm$ SD) intracellular calcium baselines [nM/mg protein] before and after KCl depolarization in cells treated with Amlodipine, Flunarizine, R(+) Bay K8644, Nimodipine, Nicardipine and Nifedipine versus untreated cells (n=28).

Time Intervall	0–29 sec	70–99 sec	140–169 sec
Control cells			
Treated	109 ($\pm$ 52)	121 ($\pm$ 41)	124 ($\pm$ 44)
Untreated	114 ($\pm$ 78)	142 ($\pm$ 76)	141 ($\pm$ 71)
One-tail p	0.44	0.23	0.3
SiRNA cells			
treated	108 ($\pm$ 59)	114 ($\pm$ 64)	115 ($\pm$ 58)
untreated	126 ($\pm$ 51)	162 ($\pm$ 49)	146 ($\pm$ 41)
One-tail p	0.14	0.01	0.004
One-tail p (treated control/siRNA cells)	0.48	0.38	0.36
One-tail p (untreated control/siRNA cells)	0.32	0.2	0.25

Table 3

Mean ($\pm$ SD) intracellular calcium peak concentrations [nM/mg protein] after KCl depolarization in untreated cells (n=68).

KCl Time point	30 sec	100 sec
Celltype		
Control cells	45 ($\pm$ 21)	41 ($\pm$ 16)
siRNA cells	47 ($\pm$ 20)	45 ($\pm$ 18)
One-tail p	0.34	0.14

Table 4

Mean ($\pm$ SD) intracellular calcium peak concentrations [nM/mg protein] after KCl depolarization in cells treated with Amlodipine, Flunarizine, R(+) Bay K8644, Nimodipine, Nicardipine and Nifedipine versus untreated cells (n=28).

KCl Time point	30 sec	100 sec
Control cells		
Treated	26 ($\pm$ 20)	22 ($\pm$ 9)
Untreated	38 ($\pm$ 11)	36 ($\pm$ 7)
One-tail p	0.06	0.005
SiRNA cells		
Treated	10 ($\pm$ 9)	14 ($\pm$ 3)
Untreated	33 ($\pm$ 13)	33 ($\pm$ 11)
One-tail p	0.005	0.003
One-tail p (treated control/siRNA cells)	0.12	0.03
One-tail p (untreated control/siRNA cells)	0.2	0.26

Table 5

Mean ($\pm$ SD) intracellular calcium peak concentrations [nM/mg protein] after KCl depolarization in cells treated with S(-) Bay K8644 and (+)Verapamil versus untreated cells (n=8).

KCl Time point	30 sec	100 sec
Control cells		
Treated	66 ($\pm$ 17)	54 ($\pm$ 24)
Untreated	41 ($\pm$ 12)	38 ($\pm$ 9)
One-tail p	0.05	0.19
SiRNA cells		
Treated	93 ($\pm$ 17)	87 ($\pm$ 27)
Untreated	48 ($\pm$ 0)	58 ($\pm$ 19)
One-tail p	0.08	0.06
One-tail p (treated control/siRNA cells)	0.001	0.02
One-tail p (untreated control/siRNA cells)	0.28	0.12

Table 6

Mean ($\pm$ SD) summarized (baseline + peak) intracellular calcium concentrations [nM/mg protein] after KCl depolarization in cells treated with Amlodipine, Flunarizine, R(+) Bay K8644, Nimodipine, Nicardipine and Nifedipine versus untreated cells (n=28).

KCl Time point	30 sec	100 sec
Control cells		
Treated	136 ($\pm$ 45)	143 ($\pm$ 37)
Untreated	161 ($\pm$ 68)	170 ($\pm$ 65)
One-tail p	0.24	0.2
SiRNA cells		
Treated	118 ($\pm$ 54)	128 ($\pm$ 64)
Untreated	162 ($\pm$ 68)	186 ($\pm$ 43)
One-tail p	0.004	0.003
One-tail p (treated control/siRNA cells)	0.22	0.23
One-tail p (untreated control/siRNA cells)	0.25	0.21

Table 7

Various types of calcium channel modulators without effect on baseline or peak intracellular calcium concentrations.

Drug name	Channel type	Drug name	Channel type
Amiodarone	L-type	Lanthanum Cl Heptahydrate	Non selective
Bepiridil	Non selective	Loperamide	Non selective
Capsaicin	TRPV 1	Methoxyverapamil HCl	L-type
Capsazepine	TRPV 1	Mibefradil di HCl	T-type
Calcimycin	Calcium ionophore	MRS 1845	SOC (store operated)
Dihydrocapsaicin	TRPV1	Neomycin Trisulfatesalhydrate	Non selective
(+) cis Diltiazem HCl	L-type	NNC 55-0396	T-type
DK-AH 269	If receptor	N-Oleyldopamine	L-type
Felodipine	L-type	Penfluridol	T-type
Flavoxate HCl	L-type	Pimozide	L-type
FPL 64176	L-type	Ruthenium	TRPV 1
Gabapentin	Unknown	SKF 96365	Non selective
HA 1077 di HCl	Non selective	Thio NAD	Calmoduline induced
6-Iodonor dihydrocapsaicin	TRPV 1	Thioridazine HCl	D2
Isradipine	L-type	R(+) Verapamil mono HCl	L-type
Icilin	TRPM 8	Zatebradine HCl	If receptor