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Original Paper

Inhibition of Lithium Sensitive Orai1/STIM1 Expression and Store Operated Ca^{2+} Entry in Chorea-Acanthocytosis Neurons by NF-κB Inhibitor Wogonin

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Key Words
Chorein • Neurons • Lithium • NFκB • Orai1 • SOCE

Abstract
Background/Aims: The neurodegenerative disease Chorea-Acanthocytosis (ChAc) is caused by loss-of-function-mutations of the chorein-encoding gene VPS13A. In ChAc neurons transcript levels and protein abundance of Ca^{2+} release activated channel moiety (CRAC) Orai1 as well as its regulator STIM1/2 are decreased, resulting in blunted store operated Ca^{2+}-entry (SOCE) and enhanced suicidal cell death. SOCE is up-regulated and cell death decreased by lithium. The effects of lithium are paralleled by upregulation of serum & glucocorticoid inducible kinase SGK1 and abrogated by pharmacological SGK1 inhibition. In other cell types SGK1 has been shown to be partially effective by upregulation of NFκB, a transcription factor stimulating the expression of Orai1 and STIM. The present study explored whether pharmacological inhibition of NFκB interferes with Orai1/STIM1/2 expression and SOCE and their upregulation by lithium in ChAc neurons. ***Methods:*** Cortical neurons were differentiated from induced pluripotent stem cells generated from fibroblasts of ChAc patients and healthy volunteers. Orai1 and STIM1 transcript levels and protein abundance were estimated from qRT-PCR and Western blotting, respectively, cytosolic Ca^{2+}-activity ($[Ca^{2+}]_i$) from Fura-2-fluorescence, SOCE from increase of $[Ca^{2+}]_i$ following Ca^{2+} re-addition after Ca^{2+}-store depletion with sarco-endoplasmatic Ca^{2+}-ATPase inhibitor thapsigargin (1μM), as well as CRAC current utilizing whole cell patch clamp

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recording. ***Results:*** Orai1 and STIM1 transcript levels and protein abundance as well as SOCE and CRAC current were significantly enhanced by lithium treatment (2 mM, 24 hours). These effects were reversed by NFκB inhibitor wogonin (50 μM). ***Conclusion:*** The stimulation of expression and function of Orai1/STIM1/2 by lithium in ChAc neurons are disrupted by pharmacological NFκB inhibition.

Introduction

Chorein binds to and supports activation of phosphoinositide-3-kinase (PI3K)-p85-subunit thus participating in the regulation of actin polymerization and cell survival [1-4]. Loss-of-function-mutations of the chorein encoding gene VPS13A (vacuolar protein sorting-associated protein 13A) result in chorea-acanthocytosis (ChAc) [5, 6], a neurodegenerative disease with hyperkinetic movements, epilepsy, impaired cognitive functions, myopathy, and erythrocyte acanthocytosis [5, 7-9]. The progressive neurodegeneration eventually leads to severe disability and early death [9]. Erythrocyte shape changes [10], neuronal apoptosis [11] and altered behaviour [11] are similarly observed in gene targeted mice lacking functional chorein.

Chorein expression is observed in a wide variety of tissues and across a wide range of cell types [12-14]: Chorein-sensitive functions include dopamine release [15], platelet activation [14], cytoskeletal architecture [16], endothelial cell stiffness [13], as well as survival of tumour cells [3], neurons and skeletal muscle cells [5, 17].

Cell survival and cell death are sensitive to alterations of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [18, 19]. $[Ca^{2+}]_i$ could be increased by Ca^{2+} release from intracellular stores with subsequent store-operated Ca^{2+} entry (SOCE) through the pore-forming Ca^{2+} channel subunits Orai1, Orai2 and/or Orai3 [20]. The Orai isoforms are activated following store depletion by the Ca^{2+} sensing proteins STIM1 and/or STIM2 [21-23]. Orai1 and SOCE are up-regulated by PI3K dependent signaling and thus sensitive to chorein [24]. Stimulators of Orai1 expression and SOCE include lithium [25-27], which may slow neurodegeneration [28-30].

Orai1 expression is up-regulated by serum & glucocorticoid inducible kinase SGK1 [31, 32] and the effect of lithium in ChAc neurons is reversed in the presence of SGK1 inhibitor GSK650394 [27]. SGK1 is partially effective by NFκB dependent up-regulation of Orai1 expression and by inhibition of Nedd4-2 triggered degradation of Orai1 protein [31, 32]. As chorein deficiency impairs activation of PI3K [1-3], it should interfere with PI3K/SGK1/NFκB dependent Orai1 upregulation. The observed signaling contributes to the anti-apoptotic effect of PI3K which confers survival of a wide variety of cells including cancer cells [33-36] and neurons [37-40].

The present study explored whether the effect of lithium on neuronal Orai1 expression and SOCE in ChAc neurons requires functional NFκB. To this end, skin fibroblasts from ChAc patients were reprogrammed and differentiated to neurons and Orai1/STIM1/STIM2 transcript levels, Orai1/STIM1/STIM2 protein abundance, SOCE and CRAC currents determined without or with prior lithium treatment in the absence or presence of NFκB inhibitor wogonin.

Materials and Methods

Generation of iPSCs

The study has been approved by the Ethical Commission of the University of Tübingen (598/2011). Informed consent was obtained from all participants and/or their legal guardian/s. Human dermal fibroblasts were isolated from ChAc patients (n = 3) and healthy volunteers (n = 3). Dermal fibroblasts were cultivated in fibroblast medium, consisting of DMEM (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Life technologies, Thermo Fisher Scientific, Waltham, Massachusetts) and 1% L-Glutamine (Biochrom). Induced pluripotent stem cells (iPSCs) were generated following a protocol

published previously [41], with minor modifications. In brief, $1x10^5$ fibroblasts were electroporated (Nucleofector 2D, Lonza) with a total of 1 µg per plasmid carrying the sequences for hOCT4, hSOX2, hKLF4, hL-MYC and hLIN28. After cultivation in fibroblast medium for 1 day, 2 ng/ml FGF-2 (Peprotech) was supplemented to the medium. From day 3 on, cells were cultivated in Essential 8 (E8) medium containing 100 µM NaB (Sigma-Aldrich). The iPSC colonies were picked manually after 3 – 4 weeks and further expanded in Matrigel coated 6-well plates. At passage 7 – 10, iPSCs were characterized and frozen in E8 medium supplemented with 40% KOSR (Thermo Fisher Scientific), 10% DMSO (Sigma-Aldrich), and 1 µM Y-27632 (Selleckchem, Munich, Germany). Characterization of generated iPSCs included genomic validation via exclusion of plasmid-integration, SNParray analysis for genetic integrity, and resequencing of mutation site, as well as functional validation via confirmation of expression of pluripotency marker, and verification of the *in vitro* differentiation potential as described previously [42]. The opportunity to apply iPSC-derived neurons to model neurological disorders is a powerful tool to identify disease-relevant alterations in patient-specific neuronal cell types (reviewed in: [43]). A careful characterization of generated iPSCs as well as the establishment of a robust and reliable protocol to generate neurons is essential to provide consistent phenotypes. Additionally, using multiple patient and control cell lines is crucial to confirm the identified cellular phenotypes. In the present study we addressed these points by genomic and functional validation of all generated iPSCs, applying a standardized neuronal differentiation protocol yielding ß-III-tubulin/CTIP-2 positive neurons as well as verifying the observed phenotypes in three independent patient lines.

Neuronal differentiation and treatment of iPSCs

Cortical neurons were generated as described previously [44]. Briefly, neural induction of iPSCs was achieved by addition of dual SMAD inhibitors (10 µM SB431542 (Sigma-Aldrich) and 500 nM LDN-193189 (Sigma-Aldrich)) to 3N medium. Cells were collected at day 10 and further expanded by cultivation in 3N medium supplemented with 20 ng/ml FGF-2 for 2 days. From day 12 on, cells were cultivated in 3N medium with medium change every other day. Cell cultures were passaged at day 27 and replated appropriately for the specific assay (RNA/Protein isolation: $5x10^5$ cells per cm^2; FACS analysis: $2.5x10^5$ per cm^2; Ca^{2+} measurements: $5x10^4$ per cm^2). Where indicated, 2 mM lithium (Sigma-Aldrich) and/or 50 µM wogonin (Sigma-Aldrich) was added to the medium 24 hours prior to measurements. Analysis was performed between day 37 and 41.

Quantitative PCR

Transcript levels of Orai1, STIM1, STIM2 and house-keeping GAPDH were determined by RT-PCR as described previously [24, 45]. Total RNA was extracted in TriFast (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After DNAse digestion reverse transcription of 2 µg RNA was performed using GoScript™ Reverse Transcription System (Promega, Hilden, Germany) according to the manufacturer's protocol. Real-time polymerase chain reaction (RT-PCR) amplification of the respective genes were set up in a total volume of 20 µl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega, Hilden, Germany) according to the manufacturer's protocol. Cycling conditions were as follows: Initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 68°C for 20 seconds. For amplification the following primers were used (5`->3`orientation):

GAPDH:
fw: TGAGTACGTCGTGGAGTCCAC;
rev: GTGCTAAGCAGTTGGTGGTG
Orai1:
fw: CGTATCTAGAATGCATCCGGAGCC;
rev: CAGCCACTATGCCTAGGTCGACTAGC
STIM1:
fw: CCTCGGTACCATCCATGTTGTAGCA
rev: GCGAAAGCTTACGCTAAAATGGTGTCT
STIM2:
fw: CAAGTTGCCCTGCGCTTTAT
rev: ATTCACTTTTGCACGCACCG

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, Munich, Germany) and all experiments were done in duplicate. The house-keeping gene GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was amplified to standardize the amount of sample RNA.

Western Blotting

Protein abundance of Orai1, STIM1/2 and GAPDH was determined by Western blotting as described previously [24, 45]. To this end, cells were centrifuged for 5 minutes at 240 g and 4°C. The pellet was washed twice with ice cold PBS and suspended in 40 µl ice-cold RIPA lysis buffer (Thermo Fisher Scientific, USA) containing Halt Protease and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, USA). Protein concentration was determined using the Bradford assay (BioRad, München, Germany). 100 µg of protein were solubilized in sample buffer at 95ºC for 10 min. The proteins were separated by 10% SDS-PAGE in Glycine-Tris buffer and electro-transferred onto nitrocellulose membranes for 90 min. After blocking with 5% milk in TBST at room temperature for 1 h, the membranes were incubated with primary anti-ORAI1 antibody (1:1000, Proteintech), anti-STIM1 antibody (1:1000, Cell Signaling), anti-STIM2 antibody (1:1000, Cell Signaling) and anti-GAPDH antibody (1:1000, Cell Signaling) at 4°C overnight. After washing (TBST), the blots were incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase (1:2000, Cell Signaling) for 1 h at room temperature. Protein bands were detected after additional washes (TBST) with an ECL detection reagent (Amersham, Freiburg, Germany) and quantified with Quantity One Software (BioRad, München, Germany). To assign the right protein size we used Protein-Marker VI (Peqlab, Erlangen, Germany).

Ca^{2+} measurements

Fura-2 fluorescence was taken as a measure of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), as described previously [24, 45]. For this purpose, cells were loaded with Fura-2/AM (2 µM, Invitrogen, Goettingen, Germany) for 20 min at 37°C. Cells were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40×/1.30 oil) built in an inverted fluorescence microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). Cytosolic Ca^{2+} activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca^{2+} removal and subsequent Ca^{2+} re-addition in the presence of thapsigargin (1 µM, Invitrogen). For quantification of Ca^{2+} entry, the slope (delta ratio/s) and peak (delta ratio) were calculated following re-addition of Ca^{2+}.

Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 Na_2HPO_4, 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca^{2+}-free conditions, experiments were performed using Ca^{2+}-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 Na_2HPO_4, 32 HEPES, 0.5 EGTA, 5 glucose, pH 7.4.

Patch clamp

Ca^{2+} release activated channel (CRAC) currents (I_{CRAC}) were determined by whole cell patch clamp recording at room temperature in voltage-clamp, fast whole cell mode. Cells were continuously superfused through a flow system inserted into the dish. The bath was grounded via a bridge filled with the external solution. Borosilicate glass pipettes (2- to 4-MΩ resistance; Harvard Apparatus, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany), were used in combination with a MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Lambrecht, Germany) and analyzed with Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, NY). Currents were recorded at an acquisition frequency of 10 kHz and 3 kHz low-pass filtered. The pipette solution contained (in mM/l): 35 NaCl, 120 CsCl, 10 HEPES/CsOH, 10 ethylene glycol tetraacetic acid (EGTA) and 0.04 inositol 1, 4,5-triphosphate ($Ins(1,4,5)P_3$, Enzo Life Sciences), pH 7.4. The external solution contained (in mM/l) 140 NaCl, 5 KCL, 10 $CaCl_2$, 20 glucose, 10 HEPES/NaOH, pH 7.4 [46].

Statistics

Data are expressed as arithmetic means ± SEM. Statistical analysis was made by unpaired t-test or Mann-Whitney test, as appropriate. $p < 0.05$ was considered as statistically significant.

Results

ChAc neurons were incubated for 24 hours in the absence or presence of 2 mM lithium. The Orai1 and STIM1/2 mRNA abundance was subsequently determined using qRT-PCR. As displayed in Fig. 1, Orai1 and STIM1/2 transcript levels were in ChAc neurons significantly increased by treatment with 2 mM LiCl. The effect of lithium on Orai1 and STIM1/2 transcript levels was abrogated by additional treatment with the NFκB inhibitor wogonin (50 μM). In the presence of lithium and wogonin, the Orai1 and STIM1/2 transcript levels were even significantly lower than the transcript levels of untreated ChAc neurons (Fig. 1). Wogonin alone tended to decrease Orai1 transcript levels and significantly decreased STIM1/2 transcript levels (Fig. 1).

Western blotting was employed for quantification of protein abundance. As illustrated in Fig. 2, the Orai1 and STIM1 protein expressions were significantly higher in ChAc neurons following 24 hours incubation in the presence than in the absence of 2 mM lithium (Fig. 2). The additional treatment with the NFκB inhibitor wogonin (50 μM) significantly decreased the Orai1 and STIM1/2 protein abundance. In the presence of both, lithium and wogonin, the Orai1 and STIM1/2 protein abundances were not significantly different from the Orai1 and STIM1/2 protein abundances in the absence of lithium and wogonin (Fig, 2).

Altered expressions of Orai1 and STIM1 were expected to be paralleled by respective changes of store operated Ca^{2+} entry (SOCE). Fura2 fluorescence was thus employed to quantify the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). For determination of SOCE, the intracellular stores were emptied by exposure of the cells to the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (1 μM) in the absence of extracellular Ca^{2+}. Re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin resulted in a sharp increase of $[Ca^{2+}]_i$ reflecting SOCE. As shown in Fig. 3B,C, emptying the intracellular Ca^{2+} stores with thapsigargin was followed by a transient increase in $[Ca^{2+}]_i$. The increase tended to be higher in lithium treated than in untreated ChAc neurons, a difference, however, not reaching statistical significance. The increase of $[Ca^{2+}]_i$ following re-addition of extracellular Ca^{2+} in the continued presence of thapsigargin was significantly higher in lithium treated than in untreated ChAc neurons (Fig. 3D,E). Treatment with the NFκB inhibitor wogonin (50 μM) significantly decreased SOCE both, in the absence and presence of lithium. In the presence of both, lithium and wogonin, SOCE was significantly lower than in untreated ChAc neurons (Fig. 3D,E).

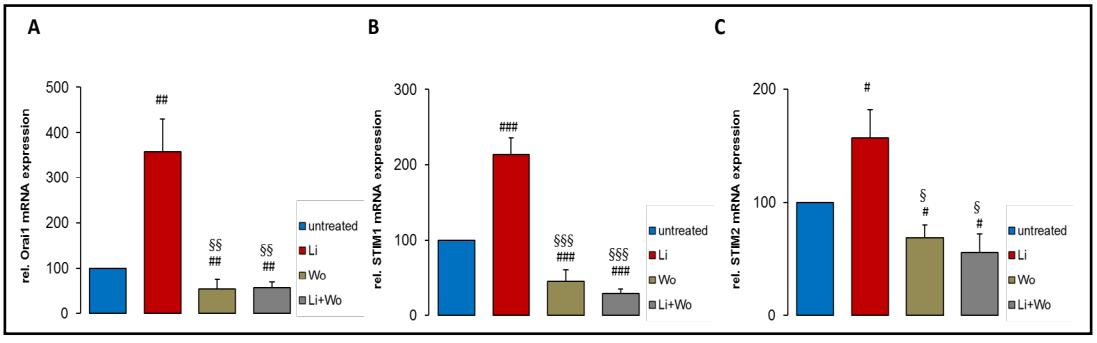

Fig. 1. Effect of lithium treatment with and without NFκB inhibitor wogonin on Orai1 STIM1 and STIM2 transcript levels in ChAc neurons. A,B,C. Arithmetic means (± SEM, n = 7) of (A) Orai1, (B) STIM1, (C) STIM2 transcript levels in neurons generated from ChAc patients without (blue or yellow bars) or with (red and grey bars) lithium (24 h, 2 mM) treatment without (blue and red bars) and with (yellow and grey bars) presence of NFκB inhibitor wogonin (50 μM). ##(p<0.01), ###(p<0.001) indicates statistically significant difference to respective value in the absence of treatments, §(p<0.05), §§(p<0.01), §§§(p<0.001) indicates statistically significant difference to respective value in the absence of wogonin.

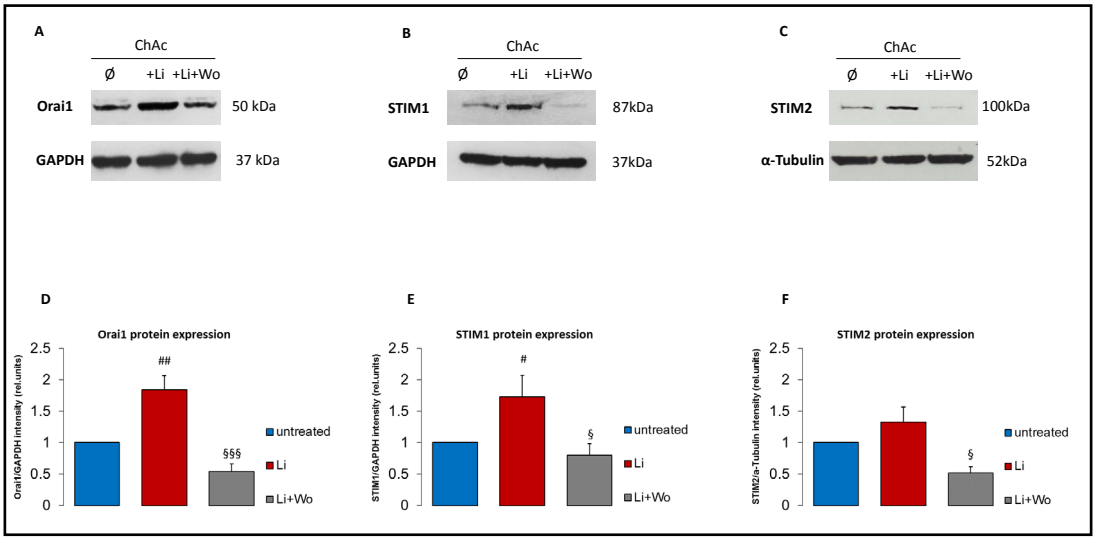

Fig. 2. Effect of lithium treatment without and with NFκB inhibitor wogonin on Orai1, STIM1 and STIM2 protein abundance in iPSC-derived neurons from ChAc patients. A,B,C. Original Western blot of (A) Orai1, (B) STIM1 and (C) STIM2 protein abundance in neurons differentiated from iPSCs derived from ChAc patients (ChAc) without or with lithium (24 h, 2 mM) treatment without and with presence of NFκB inhibitor wogonin (50 μM). D,E,F. Arithmetic means (± SEM, n = 5) of (D) Orai1, (E) STIM1 and (F) STIM2 protein abundance in neurons generated from iPSCs derived from ChAc patients without (blue bars) or with (red and grey bars) lithium (24 h, 2 mM) treatment without (red bars) and with (grey bars) presence of NFκB inhibitor wogonin (50 μM). #(p<0.05), ##(p<0.01) indicates statistically significant difference to respective value in the absence of treatment, §(p<0.05), §§§(p<0.001) indicates statistically significant difference to respective value in the absence of wogonin.

The function of Orai1 was further quantified by measurement of Ca^{2+} release activated Ca^{2+} channel (CRAC) currents (I_{CRAC}) utilizing whole cell patch clamp recording. As shown in Fig. 4, I_{CRAC} was significantly higher in lithium treated than in untreated ChAc neurons. The additional treatment with the NFκB inhibitor wogonin (50 μM) significantly decreased I_{CRAC} (Fig. 4).

Discussion

The present study sheds further light on the signaling contributing to the regulation of Orai1, STIM1 and STIM2 expression, Ca^{2+} release activated channel (CRAC) currents (I_{CRAC}) and store operated Ca^{2+} entry (SOCE) in chorea-acanthocytosis (ChAc) neurons. The results confirm the previous observations that lithium up-regulates Orai1and STIM1 expression, I_{CRAC} and SOCE in ChAc neurons [27]. Inhibition of NFκB inhibitor wogonin abrogates the stimulating effect of lithium on Orai1 and STIM1/2 transcript levels and protein abundance, SOCE and CRAC current. Wogonin significantly decreases Orai1 and STIM1/2 transcript levels and SOCE even in the absence of lithium indicating that NFκB is stimulating Orai1 and STIM1/2 expression as well SOCE independent of lithium. NFκB may thus play a permissive role for the effect of lithium. In any case, NFκB is apparently a stimulator of Orai1 and STIM1/2 expression as well SOCE in both, the presence and absence of lithium.

Stimulation of SOCE may result in oscillations of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [47] with rapid increase of $[Ca^{2+}]_i$ due to intracellular Ca^{2+} release and activation of SOCE followed by rapid decrease of $[Ca^{2+}]_i$ due to SOCE inhibition and Ca^{2+} extrusion [48]. The repeated short increases of $[Ca^{2+}]_i$ are followed by activation of Ca^{2+} dependent transcription factors and reorganization of the actin filament network without cell injury resulting from sustained increases of $[Ca^{2+}]_i$ [49, 50].

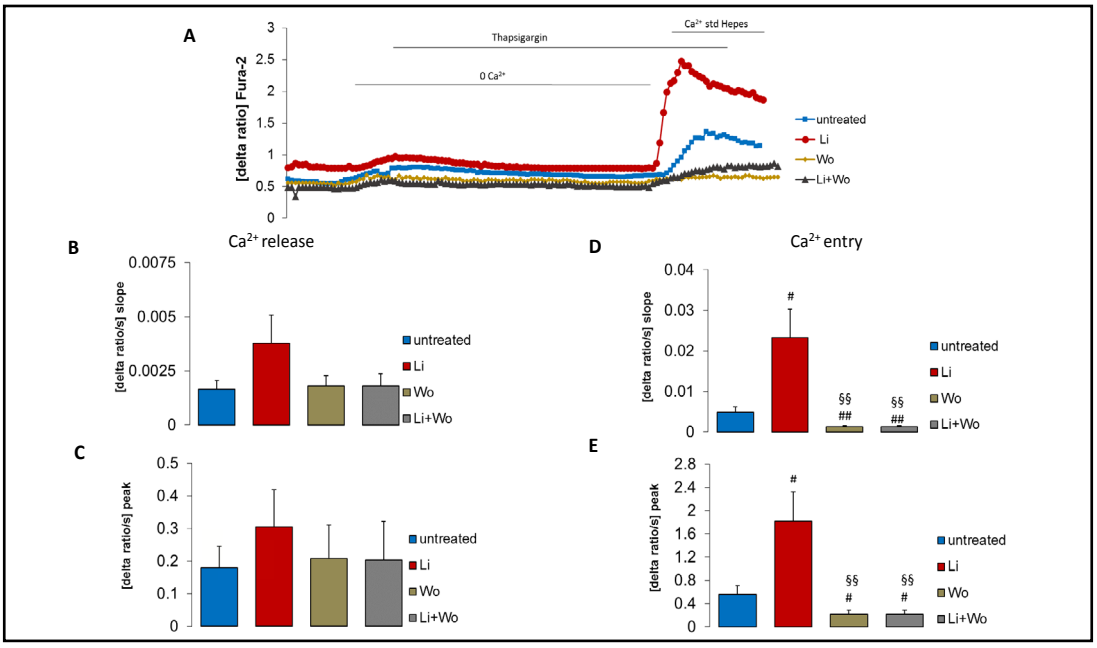

Fig. 3. Intracellular Ca^{2+} release and store-operated Ca^{2+} entry (SOCE) in iPSC-derived neurons from ChAc patients without or with lithium treatment without and with NFκB inhibitor wogonin. A. Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin (1 μM), as well as re-addition of extracellular Ca^{2+} in neurons generated from ChAc patients without (blue) and with (red) lithium (24 h, 2 mM) treatment in the absence of NFκB inhibitor wogonin as well as without (yellow) and with (grey) lithium (24 h, 2 mM) in presence of NFκB inhibitor wogonin. B,C. Arithmetic means (± SEM, n = 30-40 cells from 3 individuals) of slope (B) and peak (C) increase of fura-2-fluorescence-ratio following addition of thapsigargin (1 μM) in ChAc neurons without (blue or yellow bars) or with (red and grey bars) lithium (24 h, 2 mM) treatment without (blue and red bars) and with (yellow and grey bars) NFκB inhibitor wogonin (50 μM). D,E. Arithmetic means (± SEM, n = 30-40 cells from 3 individuals) of slope (D) and peak (E) increase of fura-2-fluorescence-ratio following re-addition of extracellular Ca^{2+} in neurons from ChAc patients without (blue or yellow bars) or with (red and grey bars) lithium (24 h, 2 mM) treatment without (blue and red bars) and with (yellow and grey bars) NFκB inhibitor wogonin (50 μM). #($p<0.05$), ##($p<0.01$) indicates statistically significant difference to respective value without treatment, §§($p<0.01$) indicates statistically significant difference to respective value in the absence of wogonin.

Ca^{2+} oscillations trigger several cellular functions [51, 52] including entry into the S and the M phase of the cell cycle [53] and cell survival [54, 55]. The Orai isoforms [20] and their regulators STIM 1 or 2 [21] confer survival, proliferation, and migration of tumor cells [56-59] and neural stem/progenitor cells [60]. In contrast to Ca^{2+} oscillations, sustained increases of cytosolic Ca^{2+} activity trigger suicidal cell death [61-63].

Lithium has previously been shown to support survival of ChAc neurons [27]. Lithium has further been shown to favorably influence other neurodegenerative diseases including Huntington´s chorea, Alzheimer´s disease, Parkinson´s disease, amyotrophic lateral sclerosis as well as spinocerebellar ataxias type 1 and type 3 [28-30, 64, 65]. Mechanisms invoked in the neuroprotective effect of lithium include direct or Akt-mediated inhibition of glycogen synthase kinase GSK-3β, Akt-mediated inhibition of the proapoptotic forkhead box class O transcription factor Foxo3a and murine double minute (MDM), stimulation of production and activity of neuroprotective brain derived neurotrophic factor BDNF, up-regulation of antiapoptotic protein Bcl-2, as well as down-regulation of proapoptotic transcription factor p53, of the proapoptotic proteins Bad and Bax, of glutamate excitotoxicity, of calpain and of oxidative stress [30, 66].

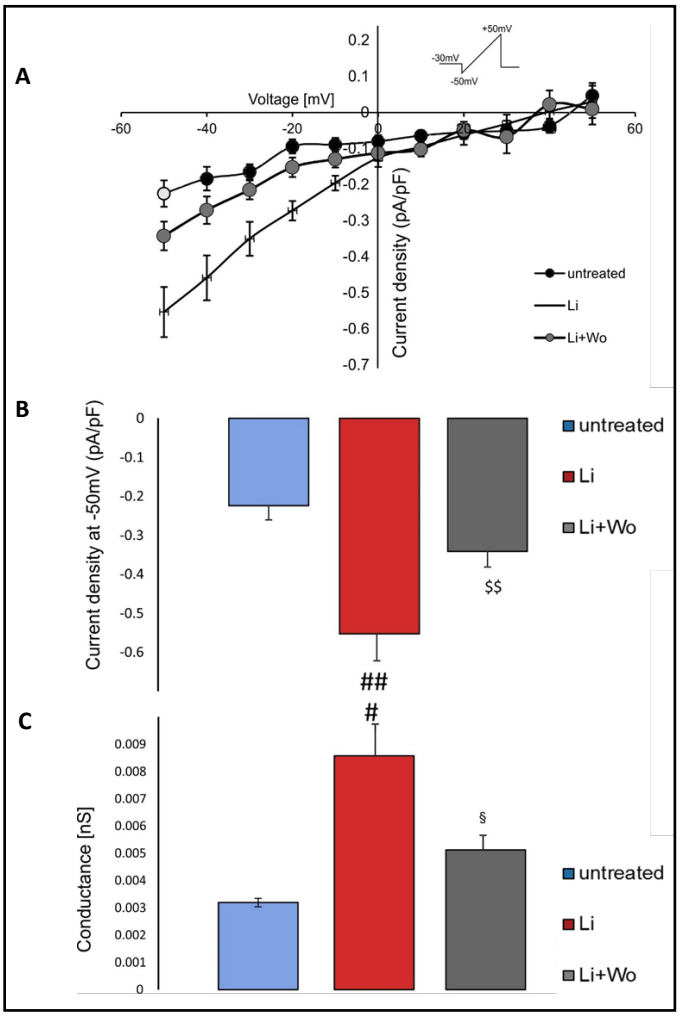

Fig. 4. Ca^{2+} release activated channel (CRAC) currents (I_{CRAC}) in iPSC-derived neurons from ChAc patients without or with lithium treatment without and with NFκB inhibitor wogonin. A. Mean current-voltage (I/V) relationships of currents normalized to cell capacitance in neurons generated from ChAc patients without (n=7) or with lithium treatment without (n=6) and with (n=8) NFκB inhibitor wogonin. The voltage protocol is shown (not to scale), whereby cells were held at -30 mV and voltage steps were applied in 10 mV increments for 200 ms from -50 mV to +50 mV. B. Mean current density (normalized by capacitance) (± SEM, n=5-7) obtained from peak of current at -50 mV without (blue bars) or with lithium (24 h, 2 mM) treatment without (red bar) and with (grey bar) presence of NFκB inhibitor wogonin (50 µM). C. Mean whole-cell conductance of inward currents (± SEM, n=5-7) calculated by linear regression of I/V curves shown in (A) between -50 mV and -10 mV without (blue bars) or with lithium (24 h, 2 mM) treatment without (red bars) and with (grey bars) presence of NFκB inhibitor wogonin (50 µM). ##(p<0.01), ###(p<0.001) indicates statistically significant difference to respective value in the absence of lithium treatment. #(p<0.05), ##(p<0.01) indicates statistically significant difference to respective value in untreated cells, §(p<0.05) indicates statistically significant difference to respective value in the absence of wogonin.

The stimulating effect of NFκB on Orai1/STIM1 expression, I_{CRAC} and SOCE observed here could, at least in theory, impact on neuronal cell survival and contribute to the beneficial effect of lithium. The present observations, however, cannot be taken as evidence for an inhibitory effect of NFκB on neurodegeneration. NFκB expressed in microglial cells has previously been shown to participate in the orchestration of neuroinflammation and thus neurodegeneration [67-69] and NF-κB inhibitors are considered as potential treatment for neurodegenerative diseases [69]. It should be further kept in mind that neurons gained by reprogramming of skin fibroblasts and kept in cell culture do not necessarily respond to challenges in an identical way as neurons in situ. Thus, caution is warranted if the present results are extrapolated to native human physiology and pathophysiology.

Conclusion

In conclusion, pharmacological inhibition of NFκB markedly decreases Orai1 and STIM1 expression as well as I_{CRAC} and store operated Ca^{2+} entry in cultured ChAc neurons. It further abrogates the stimulating effect of lithium on Orai1 and STIM1 expression as well as I_{CRAC} and

store operated Ca^{2+} entry in ChAc neurons. To which extent this effect impacts on ChAc and other neurodegenerative disorders, remains to be shown.

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B.S., S.H., L.P., Z.H., I.S., T.M., A.M. performed experiments, C.S., L.S., F.L. designed the study and share last authorship for this paper, and F.L. drafted the manuscript. All authors corrected and approved the final version of the manuscript.

Disclosure Statement

The sponsors had no role in study design, the collection, analysis and interpretation of data, in the writing of the report, and in the decision to submit the article for publication. All authors confirm that they have no competing financial interests to disclose.

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