A TRPC1/TRPC3 mediated increase in store-operated calcium entry is required for differentiation of H19-7 hippocampal neuronal cells

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Running Title: Role of TRPC proteins in differentiation of cultured hippocampal cells

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SUMMARY

Store-operated calcium entry (SOCE) and TRPC protein expression were investigated in the rat-derived hippocampal H19-7 cell line. Thapsigargin-stimulated Ba\(^{2+}\) entry and the expression of TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 mRNA and protein were observed in proliferating H19-7 cells. When cells were placed under differentiating conditions, a change in TRPC homolog expression profile occurred. The expression of TRPC1 and TRPC3 mRNA and protein dramatically increased, while the expression of TRPC4 and TRPC7 mRNA and protein dramatically decreased; in parallel a 3.4-fold increase in the level of thapsigargin-stimulated Ba\(^{2+}\) entry was observed, and found to be inhibited by 2-APB. The selective suppression of TRPC protein levels by siRNA approaches indicated that TRPC1 and TRPC3 are involved in mediating SOCE in proliferating H19-7 cells. Although TRPC4 and TRPC7 are expressed at much higher levels than TRPC1 and TRPC3 in proliferating cells, they do not appear to mediate SOCE. The co-expression of siRNA specific for TRPC1 and TRPC3 in proliferating cells inhibited approximately the same amount of SOCE as observed with expression of either siRNA alone, suggesting that TRPC1 and TRPC3 work in tandem to mediate SOCE. Under differentiating conditions, co-expression of siRNA for TRPC1 and TRPC3 blocked the normal 3.4-fold increase in SOCE, and in turn, blocked the differentiation of H19-7 cells. This study suggests that placing H19-7 cells under differentiating conditions significantly alters TRPC gene expression and increases the level of SOCE, and that this increase in SOCE is necessary for cell differentiation.
INTRODUCTION

Ca\textsuperscript{2+} is a ubiquitous second messenger, which plays a key role in regulating cellular processes such as gene expression, secretion, proliferation and apoptosis (1-3). Thus, the concentration of Ca\textsuperscript{2+} is carefully controlled through regulation of a variety of membrane channels and pumps. In excitable cells, the level of Ca\textsuperscript{2+} entry is controlled primarily by well characterized voltage-gated channels, as well as a variety of ligand-gated channels (4,5). In contrast, the principal mechanism for regulating Ca\textsuperscript{2+} entry in non-excitable cells is via non-voltage-gated channels, such as store-operated channels (SOCs) and receptor-operated channels (ROCs). SOCs are activated by emptying of the intracellular inositol trisphosphate (InsP\textsubscript{3})-sensitive Ca\textsuperscript{2+} stores. The resulting Ca\textsuperscript{2+} entry, initially referred to as capacitative calcium entry (6), is often called store-operated calcium entry (SOCE). The best characterized SOC is the calcium release-activated Ca\textsuperscript{2+} channel (CRAC) seen in lymphocytes and macrophages, characterized by an inward rectifying current (I\textsubscript{CRAC}) and a high selectivity for Ca\textsuperscript{2+} (7), but SOCs with different characteristics have been well documented in other cells systems (8). Although the exact molecular configuration of SOCs have not been conclusively identified, strong evidence points to the involvement of transient receptor potential (TRP) proteins in this process (9-12), although many of the same TRPC homologs have also been implicated in mediating receptor-operated channels (ROCs) (13,14).

The TRP family of proteins is a wide and diverse group, both structurally and functionally. Currently, the mammalian TRP family is divided into three large groups, the TRPC subfamily (TRPC1-TRPC7), the TRPV subfamily, and the TRPM subfamily (15). The seven mammalian TRPC proteins have been shown to be expressed in a variety of tissues, with surprisingly high levels of expression seen in the brain, an observation that raises questions concerning the role of TRPC channels in the brain. While it is clear that the type of channels...
reported to result from TRPC protein expression would be critical for the functioning of non-excitatory cells, where there are few voltage-sensitive channels expressed, it is much less clear why excitatory cells would need such channels. Excitable cells have numerous subtypes of voltage-gated Ca\textsuperscript{2+} channels, that mediate much larger currents than are carried by the SOCs characterized to date. Also, while SOCs can be influenced by membrane depolarization via a reduced driving force for Ca\textsuperscript{2+}, a membrane depolarization would result in a decrease, instead of an increase, in Ca\textsuperscript{2+} entry via SOCs. It is more likely that SOCs, and other channels formed from TRPC proteins, are neuromodulatory for neuronal cells most likely being stimulated by agonists for G protein coupled receptors (GPCRs). To pursue the importance of TRPC proteins in excitatory cells, we have investigated the expression profile of TRPC proteins, as well as the existence of SOCE, in H19-7 cells, a cell culture model for hippocampal neurons. Our results establish H19-7 cells as an important model cell line for the future study of the physiological importance of TRPC proteins and SOCE in neurons.
MATERIALS AND METHODS

Materials

Fura 2 free acid, fura 2-AM and Pluronic F-127 were purchased from Molecular Probes.

Thapsigargin was purchased from LC laboratories and G418 from Mediatech. HBSS, DMEM and Ca\(^{2+}\)-free HBSS solutions came from Gibco BRL. All other reagents were either obtained from Sigma, or their source is acknowledged when the agent is first discussed.

Cultured H19-7 hippocampal neurons

The H19-7 cell line was established in the laboratory of Dr. Bruce Wainer in collaboration with the laboratory of Dr. Marsha Rosner, according to the protocol published in Eves et al (16). In short, the cells were isolated from the hippocampus of 19 day old rat embryos. They were immortalized by transfecting with a temperature-sensitive version of SV40 large T antigen. For proliferation, the cells are cultured on poly-L-lysine coated (1 mg/ml in 50 mM boric acid) coverslips in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 
\(\mu\)g/ml streptomycin, 200 
\(\mu\)g/ml G418, and 2 mM glutamine (DMEM-Proliferation medium). The cells are grown at 33 \(^\circ\)C in humidified 5% CO\(_2\)-95% air. To initiate differentiation, the cells are incubated at 39 \(^\circ\)C and placed in N2 medium, which consists of DMEM supplemented with 1% fetal bovine serum, 50 units/ml penicillin, 50 \(\mu\)g/ml streptomycin, 200 \(\mu\)g/ml G418, 2 mM glutamine, 31.5 ng/ml progesterone, 100 \(\mu\)g/ml transferin and 30 \(\mu\)g/ml sodium selenate. Then 50 ng/ml of basic fibroblast growth factor (bFGF) is added to the N2 medium and the cells allowed to differentiate.

\(Ca^{2+}\) imaging-

\([Ca^{2+}]_i\) concentration was measured in cells loaded with the fluorescent indicator fura 2. Cells were plated onto poly-L-lysine-coated 25-mm coverslips. On the morning of the
experiment, cells were washed twice with HEPES-buffered HBSS, loaded for 1 hour with 5 μm fura 2-AM in HBSS supplemented with 1 mg/ml BSA + 0.025% Pluronic F127, and then unloaded in HBSS for another 30 minutes. The coverslips were mounted as the bottom of a chamber that was placed on the stage of a Nikon Diaphot inverted epifluorescence microscope. Cells in the chamber were perfused via an eight-channel syringe system. A suction pipette maintained a constant volume of solution (~ 0.5 ml) in the chamber.

An InCyt IM2™ fluorescence imaging system (Dual-wavelength fluorescence imaging system, Intracellular Imaging Inc., Cincinnati, OH) was used to measure [Ca$^{2+}$], during the experiment. Excitation light from a Xenon light source was alternately passed through 340 and 380 narrow pass filters mounted in a Sutter filter wheel (Lamda 10-C). The 510 nm emissions were captured by a Cooled CCD camera (Cohu 4915). The images were transmitted to a computer and processed with the imaging software InCyt IM2™ 4.6. [Ca$^{2+}$], was calculated by measuring the ratio of the two emission intensities for excitation at 340 nm and 380 nm. Calcium standard solutions, that were prepared with fura 2 potassium salt, were used to create a graph of fluorescence ratio (F340/F380) as a function of Ca$^{2+}$ concentration (nM). This graph was then used to convert fluorescence ratios in an experiment to calcium concentrations. For experiments comparing proliferating and differentiated cells, individual cells were circled and the Ca$^{2+}$ response of individual cells monitored. For the siRNA experiments in proliferating cells, a field of approximately 160 cells was monitored and an averaged response for the coverslip was recorded.

In experiments in which Ba$^{2+}$ influx was measured, the data are reported as the 340/380 ratio (F340/380) since the fura 2 calibration curve for Ba$^{2+}$ differs from the calibration curve for Ca$^{2+}$. A measure of the level of SOCE in the cells was obtained by subtracting the slope of the Ba$^{2+}$ leak (before stimulation) from the slope of Ba$^{2+}$ influx (after stimulation) for each cell.
coverslip trace.

Nominally Ca\(^{2+}\)-free HBSS was prepared by stirring Ca\(^{2+}\)-free, Mg\(^{2+}\)-free, and HCO\(_3^-\)-free HBSS with Chelex-100 beads. After filtering out the Chelex-100 beads, MgCl\(_2\) was added to a final concentration of 1 mM.

**siRNA constructs**

For each individual rat TRPC homolog, potential siRNA target sites (19 nucleotides in length) were chosen. The potential target sites were compared to the rat genome database by using BLAST (www.ncbi.nlm.nih.gov/BLAST) and any target sequences with homology to other coding sequences were eliminated from consideration. Hairpin siRNA template oligonucleotide design was done by entering siRNA target sequences into the web-based insert design tool at the following address: [www.ambion.com/techlib/misc/psilencer_converter.html](http://www.ambion.com/techlib/misc/psilencer_converter.html). Then two complementary oligonucleotides (Table 2) were synthesized, annealed, and ligated into the linearized pSilencer 3.1 H1 hygro vector (Ambion Inc.) for each siRNA target site. All procedures were performed as directed by the manufacturer's instruction manual (Ambion Inc.). The inserts were sequenced to confirm that there were no unwanted mutations. For the co-expression of siRNA for TRPC1 and TRPC3, the TRPC1 siRNA was expressed in a vector containing puromycin selectable markers and the cells stably expressing siRNA to TRPC3 were transfected with the siTRPC1 construct and grown in selection medium containing both hygromycin and puromycin to establish a stable cell line.

**Transfection**

Proliferating H19-7 cells were grown in 6 well plates to 50% confluency and transfected the next day by using GenePorter II Transfection reagent (Gene Therapy Systems Inc.). 48 hrs after
transfection 250 μg/ml of hygromycin (or hygromycin + puromycin) was added. Surviving cells after 1 week were collected and used in all experiments.

**Total RNA Isolation**

Total RNA was isolated from H19-7 cells using the RNeasy Mini Kit (QIAGEN Inc.), and treated with Dnase I (Invitrogen). The RNA sample was additionally purified by ethanol precipitation and its concentration determined by measuring absorbance at 260 nm.

**PCR primers**

Polymerase chain reaction (PCR) primers for rTRPC1 and rTRPC3-7 were designed based on published sequences in GenBank (Table 1).

**RT-PCR**

First-strand cDNA was prepared from 1 μg total RNA using SuperScript™ III RNase H- Reverse Transcriptase (Invitrogen) and 1 μg oligo-dT. The mRNA samples were denatured at 65 °C for 5 min. Reverse transcription was performed at 50 °C for 55 min and was stopped by heating samples at 75 °C for 10 min. The cDNA was amplified by PCR using the TRPC isoform-specific primers listed in Table 1 and PCR Platinum Supermix reagent (Invitrogen). PCR conditions: 95 °C for 2 min, 40 cycles of 95 °C for 15 sec, followed by 60 °C for 1 min. After completion of the 40 cycles, samples were incubated at 60 °C for 10 min. A β-actin positive control was performed alongside the experimental samples, as well as a negative control with no reverse transcriptase. The PCR products were visualized on an ethidium bromide-stained agarose gel and the bands digitized by Kodak Electrophoresis Documentation and Analysis System 120 with Kodak 1D 3.0 software for Macintosh computers.
Quantitative real-time RT-PCR (QRT-PCR)-

Real-time PCR was performed on the ABI Prism 7700 Sequence Detection Systems by using SYBR® Green PCR Core Reagents (Applied Biosystems, USA) and cDNA synthesized as described above. PCR was performed using the kit protocol in a 25 μl reaction volume. The integrity of the RT-PCR products was confirmed by melting curve analysis. Melting curves for rTRPC1, rTRPC3, rTRPC4, and rTRPC7 showed one specific signal. The amount of PCR products in differentiated H19-7 cells or in proliferating cells transformed with siRNA constructs was calculated in reference to the individual calibration curves based on cDNA obtained from proliferating H19-7 cells.

Western blotting-

H19-7 cells (proliferating and differentiated) were grown on 10 cm dishes under the conditions described above. Cells were lysed in modified RIPA buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% SDS, 1% NP40, 1% Na Deoxycholate, 2 mM EDTA, 2 mM Na₂VO₄, 2 mM Na₄P₂O₇, 2 mM NaF). The lysates were clarified by centrifugation and protein concentration was measured by BCA kit (Pierce). Total protein extract (50-150 μg) was applied on 8% SDS PAGE (16 cm x 16 cm gels) and run overnight. The proteins were transferred onto Immobilon P membrane (Millipore Inc.), and after 1 hr blocking, treated with polyclonal anti-TRPC antibodies raised against the TRPC homolog tested. The concentrations used were as follows: anti-TRPC3 - 1:200 (Alomone Labs, Israel ), anti-TRPC1 and anti-TRPC4 - 1:250 (Santa Cruz Biotechnology, USA), anti-TRPC7 - 1:500 (Abcam, UK). Membranes were washed 4 x 15 min with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), incubated for 30 min at room temperature with secondary anti-rabbit or anti-goat antibody (1:5000 in TBS-T), washed under the same conditions, and developed with SuperSignal Chemiluminescent Substrate (Pierce) at a suitable time so as not to saturate the film. The films were
digitized on a flatbed scanner and the relative spot intensities determined in Photoshop 6.0. The images were inverted, the bands outlined, and a measure of the average gray level and the number of pixels in the spot obtained within the histogram function. We used the product of the average gray level value and the number of pixels to represent the integrated signal in the band. Each Western blot was repeated at least three times using different cell lysates.
RESULTS

*Endogenous expression of TRPC homologs-*

The initial goal was to determine which TRPC homologs are expressed in proliferating H19-7 cells. For this purpose, cells were grown at 33 °C in DMEM-Proliferation medium. Cells were removed from their growth environment and mRNA extracted as described in the Methods section. RT-PCR reactions were run utilizing primers specific for individual TRPC homologs (Table I). As shown in figure 1, proliferating H19-7 cells express mRNA for TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7. Negative controls with no reverse transcriptase showed no signal.

Next it was determined whether the TRPC mRNA expression profile was modified when cells were differentiated. Therefore, cells were switched to N2 medium, shifted from 33 °C to the non-permissive temperature of 39 °C, and treated with FGF over a period of several days. Although differentiation is monitored on the basis of morphological features such as elongation and development of axonal processes (Fig. 2), differentiated H19-7 cells previously have been characterized to exhibit significantly lower rates of [3H]-thymidine incorporation than their undifferentiated counterparts, to express several uniquely neuronal/glial markers such as NFP, MAP-2, and GAP-43, and to show activation of neuronal-like sodium channels (16-18). Proliferating cells and post mitotic, differentiated cells were then assayed for TRPC mRNA expression using methods of real-time PCR. As shown in figure 3, there is a definite disparity in the mRNA levels of certain TRPC homologs in proliferating versus differentiated cells. The mRNA levels for TRPC1 and TRPC3 dramatically increase in magnitude, while the mRNA level for TRPC4 and TRPC7 dramatically decline, upon differentiation.

While the difference in mRNA levels in figure 3 suggest that a difference in TRPC protein levels might exist between proliferating and differentiated H19-7 cells, it was necessary to
demonstrate this by Western blots. Thus, Western blots were run on extracts of both proliferating and differentiated cells to monitor levels of TRPC1, TRPC3, TRPC4, and TRPC7 protein. As shown in figure 4, proliferating cells have much higher levels of TRPC4 and TRPC7 protein while differentiated cells have much higher levels of TRPC1 and TRPC3 protein.

Levels of SOCE in proliferating and differentiated H19-7 cells

To investigate the functional implications of the difference in rTRPC expression profile between proliferating and differentiated H19-7 cells, we examined the level of SOCE was examined under these two conditions. H19-7 cells were plated on poly-L-lysine coated coverslips and then treated under proliferating or differentiating conditions (see Materials and Methods section) for a period of several days. Cells were loaded with fura-2 and the intracellular Ca\(^{2+}\) concentration monitored by fluorescence imaging. Cells were incubated in HBSS and a baseline Ca\(^{2+}\) value established. In initial experiments, cells were shifted briefly to a Ca\(^{2+}\)-free HBSS medium containing Ba\(^{2+}\) to assess the basal leak. Cells were then shifted to a Ca\(^{2+}\)-free HBSS solution and thapsigargin added at a dose of 1 \(\mu\)M. Following the return of Ca\(^{2+}\) to basal values, Ba\(^{2+}\) was added and the Ba\(^{2+}\) entry monitored. Representative time courses for individual proliferating (Fig 5A, left panel) or differentiated cells (Fig 5B, left panel) are shown. One can see from this example that the Ba\(^{2+}\) entry in response to Ca\(^{2+}\) store depletion is much greater in the differentiated H19-7 cells. We chose Ba\(^{2+}\) as the entering cation as it is not pumped by the Ca-ATPases (19,20) and therefore can distinguish between effects on Ca\(^{2+}\) entry versus Ca\(^{2+}\) pump pathways. A statistical analysis of the Ba\(^{2+}\) entry measurements is shown in figure 5C. The Ba\(^{2+}\) entry in proliferating H19-7 cells is 0.0010 +/- 0.00003 sec\(^{-1}\), while the Ba\(^{2+}\) entry in differentiated cells is 0.0034 +/- 0.00014 sec\(^{-1}\) (these values are statistically different, \(p < 0.00001\)). The basal
Ca$^{2+}$ value, the area under the TG-stimulated Ca$^{2+}$ release curve, and the Ba$^{2+}$ leak flux (essentially zero) were all comparable for proliferating versus differentiated H19-7 cells.

To assure that thapsigargin was equally depleting Ins-P$_3$-sensitive stores in proliferating versus differentiated cells, we assessed the ability of CCh to release intracellular Ca$^{2+}$ following treatment of cells with thapsigargin. Addition of 100 µM CCh has no effect on Ca$^{2+}$ release following thapsigargin treatment in either proliferating (Fig 5A, center panel) or differentiated (Fig 5B, center panel) H19-7 cells. In the absence of Ca$^{2+}$ store depletion, CCh can induces a robust release of intracellular Ca$^{2+}$ in both proliferating (Fig 5A, right panel) and differentiated (Fig. 5B, right panel) cells.

The question arose as to whether the elevation of SOCE in differentiated H19-7 cells would show up as an increase in agonist-stimulated Ca$^{2+}$ entry. Thus, in Figure 6, Ba$^{2+}$ entry was monitored following the stimulation of cells in 100 µM CCh. The rate of Ba$^{2+}$ entry was dramatically elevated in differentiated cells (Fig 6B) in comparison to proliferating cells (Fig 6A). The increase was statistically significant (Fig 6C, p < 0.0001).

It seemed possible that the higher SOCE in differentiated versus proliferating H19-7 cells could be the result of an increase in membrane potential in the differentiated cells. While store-operated channels (SOCs) are not regulated by membrane potential, the Ca$^{2+}$ entry via these channels is sensitive to the electrical driving force of the membrane potential. To determine whether this was the case, an approach was adopted that had been used to answer a similar question of membrane potential involvement in vasopressin-stimulated Ca$^{2+}$ entry in cultured smooth muscle cells (21). After depletion of intracellular stores, cells were placed in a high K$^+$, Ca$^{2+}$-free HBSS medium to depolarize both proliferating and differentiated H19-7 cells and Ba$^{2+}$ added to monitor SOCE under these conditions. If the difference between proliferating and differentiated cells were eliminated by membrane depolarization, this would indicate that a
change in membrane potential was causative for the difference in SOCE. The data in figure 7 show that, even in the presence of membrane depolarization, the SOCE was markedly higher in differentiated versus proliferating H19-7 cells.

*The up-regulated SOCE is inhibited by 2-APB*

As the initial step in characterizing the pathways for TG-stimulated Ba$^{2+}$ entry in the H19-7 cells, the effect of 2-APB, a known inhibitor of SOCE, was investigated. The data in Fig 8A show the effect of several doses of 2-APB on thapsigargin-stimulated Ba$^{2+}$ entry in differentiated cells, and show that a dose of 100 μM is required to fully inhibit thapsigargin-stimulated Ba$^{2+}$ entry in H19-7 cells. A similar dose response was observed in proliferating cells (data not shown). Addition of 100 μM 2-APB produced inhibitions of TG-stimulated Ba$^{2+}$ entry of 97% in proliferating and 93% in differentiated cells (Fig 8B). Thus, the 2-APB data support the argument that SOCE is increased in differentiated H19-7 cells.

*Role of individual TRPC proteins in SOCE in proliferating H19-7 cells*

To explore the role of various TRPC proteins in mediating SOCE in proliferating H19-7 cells, we used an siRNA approach to selectively suppress mRNA levels for individual TRPC homologs. The mRNA levels were quantitatively monitored using real time PCR methods. The data in figure 9 illustrate the selective suppression of a particular TRPC mRNA by expression of siRNA specific for that TRPC homolog. Thus, levels of TRPC1, TRPC3, TRPC4 and TRPC7 mRNA could be selectively suppressed by approximately 90%. Western blots were performed to confirm that TRPC protein levels were also dramatically suppressed by the siRNA expression. The data in figure 10A illustrate that the specific reduction in mRNA for individual TRPC homologs is accompanied by a reduction in TRPC protein levels. Thus TRPC1 protein levels are
suppressed by 88.6 ± 7.5 %, TRPC3 levels by 83.1 ± 0.8 %, TRPC4 levels by 85.4 ± 11.6 % and TRPC7 levels by 98.2 ± 0.5% when siRNA specific for these homologs is expressed (Fig. 10B).

The effect of suppression of TRPC homologs on the level of SOCE was then determined by monitoring thapsigargin stimulation of Ba$^{2+}$ entry. The suppression of TRPC1 protein levels produced a 57 ± 3.4 % inhibition of thapsigargin-stimulated Ba$^{2+}$ entry indicating that TRPC1 is heavily involved in mediating SOCE in proliferating H19-7 cells (Figure 11). The suppression of TRPC3 protein levels by siRNA specific for this TRPC homolog produced a 64 ± 2.5 % reduction in thapsigargin-stimulated Ba$^{2+}$ entry, suggesting that TRPC3 also is heavily involved in mediating SOCE. In contrast to the findings for TRPC1 and TRPC3, the suppression of TRPC4 or TRPC7 protein levels had no significant effect on the level of thapsigargin-stimulated Ba$^{2+}$ entry (Fig 11), suggesting that in proliferating H19-7 cells, TRPC4 and TRPC7 do not participate in forming store-operated channels.

To investigate whether TRPC1 and TRPC3 are mediating two different Ca$^{2+}$ entry pathways, each of which contributes roughly 50% of SOCE, siRNAs specific for TRPC1 and TRPC3 were co-expressed. The data show that the levels of both TRPC1 and TRPC3 mRNA (Fig. 12A) and protein (Fig 12B) are specifically suppressed, and that the result is a 56% inhibition of SOCE (Fig. 12C). This level of inhibition is roughly equal to that seen for expression of each siRNA alone and suggests that TRPC1 and TRPC3 function together to mediate SOCE via the same pathway.

**Role of the increase in SOCE in differentiating H19-7 cells**

To investigate the physiological relevance of the increase in TRPC1 and TRPC3 expression following exposure of cells to differentiating conditions, we used an siRNA approach to block the rise in TRPC1 and TRPC3 protein expression. Cells co-expressing siRNA specific for
TRPC1 and TRPC3 (siTRPC1&3 cells) were placed under differentiating conditions. Initial observations revealed that following chronic exposure to differentiating conditions, siTRPC1&3 cells did not differentiate, but instead experienced a steady decrease in cell number. This is in contrast to proliferating siTRPC1&3 cells which show no viability problems. However, a time course comparing changes in control H19-7 cells to siTRPC1&3 cells, following a shift to differentiating conditions, revealed that the changes in cell morphology and SOCE could be observed in H19-7 cells as early as 1 day after a shift of culture conditions (Fig 13A, upper panel), allowing a comparison of control cells to siTRPC1&3 cells, which have a sufficient number of cells to monitor for up to 3 days after the shift of conditions. It was found that the co-expression of siRNA for TRPC1 and TRPC3 blocked the up-regulation of TRPC1 and TRPC3 (Fig 13B) as well as the normal 3.4-fold rise in SOCE following a shift to differentiating conditions (Fig 13C). Furthermore, blocking the rise in SOCE blocked the morphological changes normally observed following the shift in culture conditions (Fig 13A, lower panel) suggesting that the up-regulation of TRPC1 and TRPC3 and the increase in SOCE are important events in H19-7 cell differentiation.
DISCUSSION

Much of the effort devoted to investigating the functional role of TRPC proteins has been focused on cultured cells overexpressing TRPC isoforms. While this has provided data suggesting an involvement of TRPC proteins in both store-operated and receptor-operated Ca\(^{2+}\) entry pathways, it has also created a considerable level of controversy in the field. If one selects any individual TRPC isoform, there will be several studies supporting its role in store-operated Ca\(^{2+}\) entry, while several other papers will argue that it is receptor-operated, rather than store-operated. Much less effort has been directed at investigating the role of endogenously expressed TRPC proteins. Thus, only a few culture cell systems have been systematically studied with regard to the relative level of expression of mRNA and protein of the various TRPC isoforms, and in still fewer cell systems have methods been employed to reduce the endogenous expression level of protein for each of the individual TRPC isoforms. We have previously used an antisense approach to suppress TRPC protein levels in HEK cells (9,13), and are in the process of extending those studies with the use of siRNA methods to test for the involvement of TRPC isoforms in store-operated, OAG-stimulated, arachidonic acid-stimulated, and CCh-stimulated Ca\(^{2+}\) entry in HEK cells. Our previous antisense results suggested that both TRPC1 and TRPC3 are involved in mediating SOCE in HEK-293 cells (9), although siRNA experiments will be necessary to accurately assess the quantitative contributions of TRPC1 and TRPC3 to SOCE in HEK cells. On the other hand, TRPC4 is not involved in mediating SOCE, but is involved in mediating arachidonic acid-stimulated calcium entry in HEK-293 cells (13), a finding in contrast to reports for mouse TRPC4 (22). Due to the level of controversy in the TRPC field, it seemed important to test the generality of our results from the HEK cell system by examining TRPC expression profiles and the involvement of individual TRPC homologs in mediating SOCE in
another cell system, preferably one derived from a species other than human. In addition, it was important to begin an extensive study of TRPC proteins in excitable cells, where SOCE has been demonstrated (23-26), but where only a few investigations of the role of individual TRPC proteins (27-30) have been performed. The results reported in this paper are an important first step in the characterization of the role of TRPC proteins in a culture cell model for hippocampal neurons.

It was also important to determine whether the TRPC expression profile could be modified by a change in physiological state of H19-7 cells. Such a profile change would provide a valuable focus for investigations into the physiological role of individual TRPC proteins. This led us to study the H19-7 cell line which had previously been demonstrated to undergo differentiation following a temperature shift and addition of bFGF. Initial RT-PCR studies demonstrated that proliferating H19-7 cells expressed message for TRPC1, TRPC3, TRPC4, TRPC5, TRPC6, and TRPC7 (Fig 1). The expression of these TRPC isoforms in proliferating cells was confirmed by Western blots (blots for TRPC1, TRPC3, TRPC4, and TRPC7 are shown in Fig 4). Our early predictions that the TRPC expression profile might change upon differentiation were supported by the real time PCR data which indicated that there were dramatic shifts in the levels of mRNA for 4 TRPC isoforms (Fig 3). The levels of mRNA for TRPC4 and TRPC7 were seen to be high in proliferating cells and to decline dramatically upon differentiation. In contrast, the levels of mRNA for TRPC1 and TRPC3 were low in proliferating H19-7 cells and increased dramatically upon differentiation. Western blots supported the real time PCR data and confirmed that dramatic changes in protein level occur following cell differentiation (Fig 4). The protein levels for TRPC4 and TRPC7 were seen to be high in proliferating H19-7 cells and to decline dramatically upon differentiation. On the other
hand, protein levels for TRPC1 and TRPC3 were low in proliferating H19-7 cells and were seen to increase dramatically upon differentiation.

The dramatic shifts in TRPC protein expression made it important to look for changes in various Ca\(^{2+}\) entry pathways following differentiation, and this investigation began with a measure of SOCE in proliferating and differentiating H19-7 cells. Our early prediction was that SOCE would be higher in proliferating than differentiating cells, with the thought that these channels might be more involved with neuronal development than with function in adult neurons. To our surprise, SOCE was 3.4-fold higher in differentiated than in proliferating H19-7 cells (Fig. 5C). The observed large increase in levels of SOCE following differentiation of H19-7 cells suggests that store-operated channels may have a larger role in the physiology of excitable cells than we initially thought. While the shifts in TRPC protein expression do not provide definitive evidence for the involvement of any particular TRPC isoform in forming SOCs in H19-7 cells, they do provide some interesting hypotheses to test. First, the fact that the TRPC4 protein level declines dramatically at a time when SOCE is increasing by 3.4 fold suggests that the TRPC4 isoform does not participate in this dramatic increase in SOCE. This observation is important because there is a considerable literature arguing that mouse TRPC4 mediates store-operated Ca\(^{2+}\) entry (22,31). Second, the observation that TRPC1 and TRPC3 protein levels increase dramatically following differentiation of H19-7 cells, suggests that these proteins are likely candidates for mediating the 3.4-fold increase in SOCE seen upon differentiation.

siRNA constructs specific for the four TRPC homologs whose levels are altered upon differentiation were designed to test this hypothesis. The data in cells stably expressing siRNA specific for TRPC4 or TRPC7 indicate that, although TRPC4 and TRPC7 are the most abundant TRPC proteins in proliferating cells, they play little or no role in mediating SOCE. Expression of siRNA to TRPC4 results in no significant reduction in thapsigargin-stimulated Ba\(^{2+}\) entry (Fig. 5D).
A similar result was obtained with expression of siRNA specific for TRPC7. On the other hand, the siRNA experiments provided evidence for the involvement of TRPC1 and TRPC3 in SOCE in proliferating cells. The expression of siRNA specific for TRPC1 resulted in inhibition of approximately 57% of the thapsigargin-stimulated Ba\(^{2+}\) entry. Likewise expression of siRNA specific for TRPC3 resulted in inhibition of approximately 64% of the thapsigargin-stimulated Ba\(^{2+}\) entry. One explanation of these results is that there are two types of store-operated channels, one mediated by TRPC1 and one mediated by TRPC3. An alternative explanation is that TRPC1 and TRPC3 combine to form channels responsible for approximately 60% of the SOCE. The results from the experiments where siRNA for TRPC1 and TRPC3 were co-expressed and found to inhibit 56% of SOCE (Fig 12C) support the hypothesis that these protein combine to form a pathway that mediates approximately 60% of SOCE in proliferating H19-7 cells.

A role for TRPC1 and TRPC3 in SOCE is further supported by the observation that co-expression of siRNA to TRPC1 and TRPC3 blocks the dramatic increase in SOCE normally observed following the shift of H19-7 cells to differentiating conditions (Fig 13C). Furthermore, our findings suggest that the rise in SOCE is vital to the differentiation process since the morphological changes normally observed are prevented by co-suppressing TRPC1 and TRPC3 protein levels (Fig 13A). Chronic exposure of siTRPC1&3 cells to differentiating conditions causes a dramatic decrease in cell number suggesting that cells blocked from differentiating may choose a cell death pathway. Future studies will determine whether apoptosis is responsible for the decrease in cell number.

Although our findings argue strongly that TRPC1 and TRPC3 act together to mediate SOCE in H19-7 cells, such an interaction would be counter to recent findings that TRPC1 and TRPC3 do not directly combine on their own (32,33). However, other published evidence suggests that TRPC1 and TRPC3 can complex with either TRPC4 or TRPC5 to form channels.
or can directly complex when expressed in HEK cells (34,35). While the latter of these papers show that the combined overexpression of TRPC1 and TRPC3 in HEK cells forms channels that, in the absence of extracellular Ca\(^{2+}\), are constitutively active and can be further stimulated with diacylglycerol, it is not clear what their form of regulation is when physiological external Ca\(^{2+}\) levels are present (35). Future biochemical studies will be performed to try to confirm the predicted interaction of endogenous TRPC1 and TRPC3 proteins and explore the potential involvement of additional channel subunits or accessory proteins required for store dependent regulation of these channels.

In summary, our results suggest a novel role for TRPC proteins and SOCE in cell differentiation. In addition, the results reported here suggest that H19-7 cells are an excellent model for investigating the physiological role of TRPC proteins in excitable cells, as well as an important model system for investigating which TRPC proteins code for which endogenous Ca\(^{2+}\) entry pathways. The H19-7 cell system offers a rare opportunity to examine a physiological up-regulation of SOCE, where the cell is likely to increase all of the components (subunits as well as regulatory proteins) necessary to make additional functional channels. This is in contrast to investigating increases in SOCE following overexpression of TRPC isoforms, where a TRPC protein is overexpressed in the absence of a parallel increase in participating channel subunits or regulatory proteins.
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## Table 1

| mRNA   | Accession number | Orientation | Primer sequence (5’-3’)                                         | Location     |
|--------|------------------|-------------|------------------------------------------------------------------|--------------|
| rTRPC1 | AF061266         | Forward     | TGGTGAGAAATTACCTTCGGAAC                                          | 3640-3662    |
|        |                  | Reverse     | TTTTATAAGAATTTTGCCCAAAAG                                         | 3808-3830    |
| rTRPC3 | NM_021771        | Forward     | TTAATACCTTCACCATGCGGAG                                           | 2390-2411    |
|        |                  | Reverse     | GAACTCTTGGAGGCCAACAGG                                            | 2568-2588    |
| rTRPC4 | NM_053434        | Forward     | AAGGATTAGCTTCACGGGGGTG                                           | 2712-2732    |
|        |                  | Reverse     | CCTCCTCCTGGGCGGTTC                                              | 2890-2909    |
| rTRPC5 | NM_080898        | Forward     | TGAGTCGTCAGGCAAAACCGGTC                                          | 2821-2841    |
|        |                  | Reverse     | TCCTGCCACATAGAGTGCTGC                                            | 2953-2973    |
| rTRPC6 | NM_053559        | Forward     | AGAAATTTGGATTTTGGGAAG                                           | 2420-2441    |
|        |                  | Reverse     | TCCTTATCAATCTGGGCTGC                                             | 2601-2621    |
| rTRPC7 | XM_225159        | Forward     | CCTGTACTCCTACTACCAGGAGGTGC                                       | 2340-2363    |
|        |                  | Reverse     | TGGTGACATTATAACGCGGTAC                                           | 2499-2521    |
Table 2

| mRNA   | Accession number | Orientation | Hairpin oligos sequence (5’-3’). Target sequence printed in bold.                                                                                     | Target location |
|--------|------------------|-------------|---------------------------------------------------------------------------------------------------------------------------------|-----------------|
| rTRPC1 | AF061266         | Forward     | GATCCGGTGACTATTATAAGGTTTGTTCATATAATAGTCACCCTTTTTTGGAAA AGCTTTTCCAAAAAGGTTGACTATTATAATGTTTCTCTTGAAATTCCATATAATAGTCACCACCGG | 246-264         |
| rTRPC3 | NM_021771        | Forward     | GATCCACACGGAGGTTGCACCCTGTTCAAGAGACAGGTGCACCACCTCGTATTTTTTGGAAA AGCTTTTCCAAAAAUACGGAGGTTGCACCACCTCGTATGGTAG | 258-276         |
| rTRPC4 | NM_053434        | Forward     | GATCCCTACCAAGGATGAGGATTTCAAGAGACAGGTGCACCACCTCGTATTTTTTGGAAA AGCTTTTCCAAAAATACCAAGGATGAGGATCTCTTGAAACAGGATGACCACCTCGTATGGTAG | 2165-2183       |
| HTRPC7 | NM_020389*       | Forward     | GATCCCGCGAGAAGGCGACCAGCTCGTTCAAGAGACAGGACTGGTGCCCTTCGTTTTTTGGAAA AGCTTTTCCAAAAACGAGAAGGCGACCAGCTCGTTCAAGAGACAGGACTGGTGCCCTTCGCTCCCGG | 102-120         |

*We used the human TRPC7 sequence to design this hairpin construct because the rat TRPC7 sequence was not yet available.
FIGURE LEGENDS

**Figure 1: RT-PCR analysis for rat TRPCs in proliferating H19-7 cells.** Equal amounts of cDNA, prepared from total RNA of proliferating H19-7 cells, were added for each reaction. TRPC-isoform specific PCR primers (Table 1) and PCR conditions used for these experiments were the same as those used for real-time quantitative PCR analysis (see Methods). The PCR products were visualized on an ethidium bromide-stained agarose gel. Data is representative of three independent experiments.

**Figure 2: Morphology of cultured neuronal hippocampal cells.** Hippocampal H19-7 cells were plated on poly-L-lysine-coated coverslips and grown at 33°C in DMEM-Proliferation medium supplemented with 200 µg/ml G418. For differentiation, cells were grown in N2 medium to which 50 ng/ml of bFGF was added. Phase contrast images of proliferating H19-7 cells (upper panel) and differentiated H19-7 cells (lower panel) were captured using an ORCA-ER digital camera (Hamamatsu) attached to a Nikon eclipse TE200 microscope (20x phase objective).

**Figure 3: Real-time RT-PCR analyses of TRPC gene expression in proliferating versus differentiated H19-7 cells.** Real-time RT-PCR analyses were performed with the SYBR Green PCR Core Reagents (Applied Biosystems) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Bars depict the percentage of TRPC gene expression calculated by the method recommended in the ABI user manual. Data were normalized from triplicate reverse transcriptase reaction repeats. Data were calculated based on calibration curves for each primer
pair. The calibration curves were made by using cDNA from proliferating H19-7 cells in five serial dilutions (duplicate repeats).

**Figure 4: Western blot analyses of TRPC protein levels in proliferating versus differentiated H19-7 cells.** Proliferating and differentiated H19-7 cells were grown under conditions described in Figure 2. For each lane, 50 μg samples of total protein extract were applied and Western blots performed as described in the methods section. (A) Representative Western blots are shown for TRPC1, TRPC3, TRPC4 and TRPC7. (Lane 1 - proliferating cells, Lane 2 - differentiated cells). (B) Quantitative data summarizing three independent experiments for each TRPC homolog are based on the relative spot intensities. Although not seen at this protein concentration and exposure time, TRPC3 is present in proliferating H19-7 cells (see Fig 10A).

**Figure 5: SOCE measured in proliferating versus differentiated H19-7 cells.**

Hippocampal H19-7 cells were grown under proliferating or differentiating conditions as described in Figure 2. **(Left panel)** A representative time course of Ba\(^{2+}\) entry in proliferating (A) and differentiated H19-7 cells (B) are shown along with the average Ba entry under these conditions (C). In the absence of calcium, 2 mM Ba\(^{2+}\) was added to Ca\(^{2+}\)-free HBSS to measure Ba\(^{2+}\) leak. After switching to Ca\(^{2+}\)-free medium, Ca\(^{2+}\) stores were released with 1 μM thapsigargin and then 2 mM Ba\(^{2+}\) added. SOCE was determined by subtracting initial Ba\(^{2+}\) leak influx from Ba\(^{2+}\) influx following Ca\(^{2+}\) store depletion. Each trace represents a single cell response.

Differentiated H19-7 cells have statistically higher SOCE (* p < 0.00001). The number of individual cells tested for SOCE is shown in parenthesis. **(Center Panel)** The effect of 100 μM
CCh on Ca²⁺ release from intracellular stores following treatment with thapsigargin. Cells were treated with thapsigargin in a Ca²⁺-free medium as described above, and then after the 30 minute store-depletion period 100 μM CCh was added. There was no CCh-induced release of Ca²⁺ in either proliferating cells (n=150) or differentiated cells (n=96). **Right Panel:** 100 μM CCh was added to control cells in a Ca²⁺-free medium. There was a dramatic CCh-induced release of Ca²⁺ in both proliferating (n=90) and differentiated cells (n=45).

**Figure 6: CCh-induced Ba²⁺ entry in proliferating versus differentiated H19-7 cells.**

Hippocampal H19-7 cells were grown under proliferating or differentiating conditions as described in Figure 2. The time course of CCh-induced Ca²⁺ release and Ba²⁺ entry is shown for proliferating (A) and differentiated (B) H19-7 cells. In the absence of Ca, 100 μM CCh was used to release calcium. Once the [Ca²⁺] returned to the basal level, 2 mM Ba was added and Ba²⁺ entry measured. Each trace represents a single cell. (C) Statistical analysis of CCh-induced Ba²⁺ entry shows that differentiated cells have statistically higher Ba²⁺ entry than proliferating cells (p < 0.0001). The number of individual cells tested for CCh-induced Ba²⁺ entry is shown in parenthesis.

**Figure 7: Effect of membrane depolarization on SOCE in proliferating versus differentiated H19-7 cells.** Hippocampal H19-7 cells were grown under proliferating or differentiating conditions as described in Figure 2. Ba²⁺ entry experiments similar to those described in Figure 5 were performed in Ca²⁺-free HBSS containing either normal [K⁺] or high [K⁺] (133 mM K⁺, with K⁺ above normal being substituted for Na⁺). Statistical analysis of the leak-subtracted Ba²⁺ entry data is plotted as percent of control. Differentiated cells have
statistically higher SOCE than proliferating cells either in the absence (* p < 0.0001) or presence (** p < 0.0001) of high [K⁺]. The number of individual cells tested for SOCE is shown in parenthesis.

**Figure 8: Inhibition of SOCE by 2-APB in proliferating versus differentiated H19-7 cells.**

Hippocampal H19-7 cells were grown under proliferating or differentiating conditions as described in Figure 2. **A:** Effect of several doses of 2-APB on TG-stimulated Ba²⁺ entry in differentiated H19-7 cells. After establishing the rate of Ba²⁺ entry as described in Fig. 5, the media was changed to one containing 2 mM Ba²⁺ plus varying doses of 2-APB and the time course continued. **B:** Statistical analysis of SOCE inhibited by 100 μM 2-APB. SOCE was reduced by approximately 97% in proliferating cells (* p < 0.0001) and 93% in differentiated cells (** p < 0.0001). The number of individual cells tested for SOCE is shown in parenthesis.

**Figure 9: Real-time PCR analysis of the TRPC gene expression in control or siRNA expressing H19-7 cells.** Proliferating H19-7 cells were stably transfected with pSilencer siRNA-TRPC1 (siTRPC1), siRNA-TRPC3 (siTRPC3), siRNA-TRPC4 (siTRPC4), siRNA-TRPC7 (siTRPC7), or manufacturer pSilencer Negative control plasmid (Mock as control). Real-time PCR was performed and analyzed with the same sets of primers and the conditions as described in Figure 3.
**Figure 10: Western blot analysis of TRPC proteins in control or siRNA expressing H19-7 cells.** Proliferating H19-7 cells were stably transfected with siRNAs specific for various TRPC homologs as described in Figure 9. (A) Representative Western blots for each TRPC homolog are shown. In these experiments, samples of the total protein extract were applied to individual lanes - TRPC1 (150 μg), TRPC3 (150 μg), TRPC4 (50 μg) and TRPC7 (50 μg). (B) Data from three independent experiments for each TRPC homolog are summarized based on the relative spot intensities, and are plotted as percent of controls.

**Figure 11: Effect of TRPC siRNA expression on SOCE in proliferating H19-7 cells.** Proliferating H19-7 cells were stably transfected with siRNAs specific for various TRPC homologs as described in Figure 9. Ba\(^{2+}\) entry experiments were performed as described in Figure 5. Statistical analysis of the leak-subtracted Ba\(^{2+}\) entry (SOCE) is plotted as percent of control. Expression of siTRPC1 reduced SOCE by 57% (* p=0.004), whereas siTRPC3 expression reduced SOCE by 64% (** p=0.002), compared to their corresponding controls. There is no statistically difference between cells expressing siTRPC4 or siTRPC7 and the corresponding control cells. The number of coverslips tested for SOCE are shown in parenthesis, with each experimental point representing the averaged response from approximately 500 cells.
Figure 12. Effect of co-expressing siRNA for TRPC1 and TRPC3 on SOCE in proliferating H19-7 cells. Proliferating H19-7 cells stably co-expressing siRNA specific for TRPC1 and TRPC3 (siTRPC1&3) were made as described in Methods. A: Real-time RT-PCR analysis of TRPC mRNA expression levels was performed in control (mock) and siTRPC1&3 cells. The amount of mRNA in different cell lines (three independent repeats) was calculated in reference to the calibration curve based on mRNA obtained from non-transfected H19-7 cells. (* p<0.001). B: Western blots of the TRPC1 and TRPC3 proteins expression levels observed in proliferating control and siTRPC1&3 cells, and the corresponding statistical analysis, in which the controls were plotted as 100%, are shown. Data were obtained from at least two independent experiments. (* p<0.001). C: Ba$^{2+}$ entry experiments similar to those described in figure 5 were performed and showed that co-expressing siTRPC1&3 suppressed SOCE by approximately 56% which represents a statistically significant change (* p<0.0001). The number of cells tested for SOCE is shown in parenthesis.

Figure 13. Effect of co-expressing siRNA for TRPC1 and TRPC3 on SOCE and differentiation in H19-7 cells. The siTRPC1&3 cell line co-expressing siRNA specific for both TRPC1 and TRPC3 was established as described in Methods. The cells were placed under differentiating condition as described in Figure 2 for a maximal of 3 days. A: Images of cells were captured as described in Figure 2. While the control H19-7 cells demonstrated significant morphologic changes as early as 1 day after being placed under differentiating conditions, siTRPC1&3 cells showed little morphological change and decreased in cell number with each day. B: Real-time RT-PCR analysis of TRPC1 and TRPC3 mRNA expression levels in control (mock) and siTRPC1&3 cells placed under differentiating conditions. The amount of mRNA in
different cell lines (three independent repeats) was calculated in reference to the calibration curve based on mRNA obtained from non-transfected H19-7 cells (* p<0.001). C: Ba\textsuperscript{2+} entry experiments similar to those described in figure 5 were performed. The effects of co-expressing siRNA specific for both TRPC1 and TRPC3 on SOCE were measured in control cells and siTRPC1&3 cells which had been under differentiating conditions for 1 day. Under differentiating conditions, co-expressing siRNA to TRPC1 and TRPC3 blocked the dramatic rise in SOCE seen in control cells (* p<0.0001). The number of cells tested for SOCE is shown in parenthesis.
Figure 1
Figure 2

Proliferating

Differentiated
Figure 3

Bar graphs showing the percent of control for TRPC1, TRPC3, TRPC4, and TRPC7 mRNA levels in proliferating and differentiated cells.
Figure 4

A

Proliferating  Differentiated

Anti-TRPC1

Anti-TRPC3

Anti-TRPC4

Anti-TRPC7

B

TRPC1 protein

Percent of control

TRPC3 protein

Percent of control

TRPC4 protein

Percent of control

TRPC7 protein

Percent of control

Proliferating  Differentiated

Proliferating  Differentiated

Proliferating  Differentiated

Proliferating  Differentiated
Figure 5

A  Proliferating cells

B  Differentiated cells

C

![Graphs and figures showing calcium influx](image-url)
Figure 6

A Proliferating cell

B Differentiated cell

C

Differentiated (n=58)
Proliferating (n=66)

*
Figure 7

Bar graph showing the influx of $\text{Ba}^{2+}$ in Normal K$^+$ and High K$^+$ conditions. The graph compares proliferating and differentiated states, with the following data:

- **Normal K$^+$**
  - Proliferating (n=116)
  - Differentiated (n=72) *significance indicated by *

- **High K$^+$**
  - Proliferating (n=237)
  - Differentiated (n=141) **significance indicated by **

The y-axis represents the influx of $\text{Ba}^{2+}$, and the x-axis lists the cell states and numbers.
Figure 8

A

F340/F380

TG

2 mM Ba^{2+}

2-APB

Control

2-APB 20 µM

2-APB 50 µM

2-APB 100 µM

Time (min)

30

31

32

33

34

35

36

B

Ba^{2+} influx

Control

2-APB 100 µM

Proliferating
(n=50)

Differentiated
(n=54)

Proliferating
(n=79)

Differentiated
(n=48)

* **

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Figure 10

A

Mock  siTRPC

Anti-TRPC1

Anti-TRPC3

Anti-TRPC4

Anti-TRPC7

B

TRPC1 protein

TRPC3 protein

TRPC4 protein

TRPC7 protein

Percent of control

Percent of control

Percent of control

Percent of control

0  20  40  60  80  100  120

Mock  siTRPC1

Mock  siTRPC3

Mock  siTRPC4

Mock  siTRPC7
Figure 11

SOCE

Control (n=13)  siTRPC1 (n=15)  *

Control (n=9)  siTRPC3 (n=12)  **

SOCE

Control (n=10)  siTRPC4 (n=8)

Control (n=16)  siTRPC7 (n=16)
Figure 12

A

TRPC1 mRNA

![Bar chart showing mRNA levels for different conditions with significance levels indicated.

TRPC3 mRNA

![Bar chart showing mRNA levels for different conditions with significance levels indicated.

B

Mock siTRPC1&3

![Western blot images for TRPC1 and TRPC3 proteins.

TRPC1 protein

![Bar chart showing protein levels for different conditions with significance levels indicated.

TRPC3 protein

![Bar chart showing protein levels for different conditions with significance levels indicated.

C

Ba\(^{2+}\) influx

![Bar chart showing Ba\(^{2+}\) influx for different conditions with significance levels indicated.

Control (n=121)

siTRPC1&3 (n=61)
Figure 13

A

| Day1 | Day2 | Day3 |
|------|------|------|
| Control | siTRPC1&3 |
| ![Control](image1) | ![siTRPC1&3](image2) |
| ![Control](image3) | ![siTRPC1&3](image4) |
| ![Control](image5) | ![siTRPC1&3](image6) |

B

| TRPC1 mRNA | TRPC3 mRNA |
|------------|------------|
| Percent of control | Percent of control |
| ![Mock](image7) | ![siTRPC1&3](image8) |
| ![Mock](image9) | ![siTRPC1&3](image10) |

C

| Ba²⁺ influx |
|------------|
| Control | siTRPC1&3 |
| ![Control](image11) | ![siTRPC1&3](image12) |

*Significant difference compared to control.
A TRPC1/TRPC3 mediated increase in store-operated calcium entry is required for differentiation of H19-7 hippocampal neuronal cells
Xiaoyan Wu, Tatiana K. Zagranichnaya, Grzegorz T. Gurda, Eva M. Eves and Mitchel L. Villereal

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