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A TRPC1-mediated increase in store-operated Ca^{2+} entry is required for the proliferation of adult hippocampal neural progenitor cells

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ABSTRACT

Adult hippocampal neurogenesis plays an important role in brain function and neurological diseases. Adult neural progenitor cell (aNPC) proliferation is a critical first step in hippocampal neurogenesis. However, the mechanisms that modulate aNPC proliferation have not been fully identified. Ample evidence has demonstrated that cell proliferation is dependent on the intracellular Ca^{2+} concentration. We hypothesized that store-operated Ca^{2+} channels (SOCs), which are ubiquitously expressed in all cell types, participate in aNPC proliferation. We found that store-operated Ca^{2+} entry (SOCE) was involved in the proliferation of aNPCs and that 2-APB, Gd^{3+} and SKF96365, antagonists of SOCE and canonical transient receptor potential (TRPC), respectively, inhibited the increase in SOCE and aNPC proliferation. We therefore analyzed the expression of TRPCs in aNPCs and showed that TRPC1 is the most significantly upregulated member under proliferative conditions. Interestingly, knockdown of TRPC1 and using an antibody against TRPC1 markedly reduced the degree of SOCE and aNPC proliferation. In parallel, we observed the suppression of aNPC proliferation was found to be associated with cell cycle arrest in G0/G1 phase. Furthermore, gene expression microarray analysis revealed a selective up- or downregulation of 10 genes in aNPCs following TRPC1 silencing. Knockdown of Orai1 or STIM1 also induced a significant inhibition of SOCE and proliferation in aNPCs, and all three proteins were colocalized in the plasma membrane region of cells. Together, these results indicate that SOCE represents a principal mechanism regulating the proliferation of aNPCs and that TRPC1 is an essential component of this pathway. This discovery may be important in improving adult hippocampal neurogenesis and treating cognitive deficits.

1. Introduction

Adult hippocampal neurogenesis is a unique form of neural circuit plasticity that results in the generation of new neurons in the dentate gyrus throughout life [1]. Increasing evidence supports the idea that neurogenesis is critically involved in adult hippocampus function and might also be involved in neurological diseases [2–4]. The cellular basis for adult neurogenesis is adult neural progenitor cells (aNPCs), which exhibit the two essential properties of stem cells: self-renewal and multipotency [5]. Therefore, the proliferation of aNPCs is a key event in adult neurogenesis; however, the exact mechanisms of aNPC proliferation are unclear.

Ca^{2+} is a ubiquitous second messenger that plays key roles in the regulation of cellular processes such as gene expression, secretion and apoptosis [6]. Ca^{2+} channels are of particular interest in cell proliferation because of the profound anti-proliferative effect seen when extracellular Ca^{2+} is removed [7]. Evidence from studies of many cell types indicates that Ca^{2+} entry mechanisms have an essential role in this effect [8]. The concentration of Ca^{2+} is carefully controlled through the regulation of a variety of membrane channels and pumps. Store-operated Ca^{2+} channels (SOCs), which are expressed ubiquitously in all cell types, are thought to be critical in the regulation of a variety of cellular functions, including cell growth [9]. Ca^{2+} entry through SOCs, initially referred to as capacitative Ca^{2+} entry [10], is often called store-operated Ca^{2+} entry (SOCE).

Although the exact molecular configuration of SOCs has not been conclusively identified, some evidence points to the involvement of transient receptor potential (TRP) proteins in this process [11,12]. The TRP family of proteins is a wide and diverse group, both structurally and functionally. To date, the TRP channels identified as possibly being involved in SOCE belong to the canonical TRP (TRPC) and vanilloid TRP (TRPV) subfamilies [13]. It has recently been reported that TRPC5 plays a major role in the change of SOCE during the neuronal differentiation of $A2B5^+$ NPCs [14].

Abbreviations: Anpc, adult neural progenitor cell; BrdU, 5-bromo-2′-deoxyuridine; NPC, neural progenitor cell; SGZ, subgranular zone; SOC, store-operated Ca^{2+} channel; SOCE, store-operated Ca^{2+} entry; TG, thapsigargin; TRPC, canonical transient receptor potential; TRPV, vanilloid TRP; TuJ1, β-III tubulin.

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However, little is known about the contribution of SOCE, particularly regarding the physiological function of TRP in the proliferation of aNPCs.

We sought to determine whether SOCE participates in aNPC proliferation and, if so, what the underlying mechanism is. Herein, we report the novel findings that SOCE indeed influences the number of proliferating aNPCs and that endogenous TRPC1 is a necessary component of this process. TRPC1 knockdown induced cell cycle arrest in G0/G1, and 10 cell cycle genes were significantly up- or downregulated, indicating that these genes may mediate the effects of TRPC1 on aNPC proliferation. These findings provide a new mechanism for the modulation of the biological properties of aNPCs.

2. Materials and methods

2.1. Adult hippocampal NPC culture

Neural progenitors were isolated and cultured from the hippocampi of adult male mice (C57BL/6J, 8–10 weeks old, purchased from the Experimental Animal Center of the Third Military Medical University, Chongqing, China) as previously described [15]. The cells were propagated in DMEM/F-12 media containing 1% N2 Supplement (Invitrogen), 20 ng/ml fibroblast growth factor-basic (FGF-2; PeproTech) and 20 ng/ml epidermal growth factor (EGF; PeproTech). We replaced half of the medium every 2–3 d. All of the experimental procedures were performed in accordance with the approved principles of laboratory animal care and the ethical treatment of animals at the Third Military Medical University. All of the culture conditions and experimental manipulations were replicated in at least three independent experiments.

To test the self-renewal capacity of the cells, we seeded aNPCs at 1×10^5 cells/ml in four-well chamber slides. We added 5-bromo-2′-deoxyuridine (BrdU, Sigma) to the cells 48 h after seeding for a 1–2 h pulse. The BrdU-treated cells were fixed and acid-treated prior to immunostaining. We performed immunostaining using antibodies against nestin (1:1000, BD Biosciences) and BrdU (1:1000, Millipore).

To analyze the multipotency of the adult NPCs, neurospheres were mechanically dissociated into single cells. These cells (2.5×10^4 cells/ml) were plated directly onto laminin-coated glass coverslips in DMEM/F-12 differentiation medium containing N2 Supplement, 1 μM all-*trans* retinoic acid (Sigma), 5 μM forskolin (Sigma) and 1% FBS. The cells were differentiated for 4 d, and fresh differentiation media was added on day 2. Immunocytochemistry was performed with various antibodies (see below) to determine the phenotypes of the aNPCs.

2.2. Ca^{2+} imaging

Cells were plated onto poly-L-lysine-coated 25 mm coverslips. The cells were loaded with the Ca^{2+}-sensitive dye fura-2 by incubation with fura-2 AM (5 μM, Molecular Probes, Inc.) and 0.01% pluronic acid in HEPES-buffered salt solution (HBSS) for 45 min at 37 °C and then incubated in fresh HBSS for a further 15 min to allow deesterification of fura-2 AM. The coverslips were subsequently mounted cell side up on the free bottom of the chamber and placed on the stage of an inverted microscope. Fura-2-loaded cells were excited at 340 nm and 380 nm, and the fluorescence emission was monitored at 510 nm using an intensified CCD camera controlled by a computer. The SOC-mediated influx of Ba^{2+} following stimulation with 1 μM thapsigargin (TG) during the change from Ca^{2+}-free conditions to 2 mM Ba^{2+} was measured as described [16]. Additionally, several doses (20, 50, 100 μM) of 2-aminoethoxydiphenylborane (2-APB), 1 μM Gd^{3+}, Ruthenium Red or SKF96365 were added to the solution. In a subset of the wells, aNPCs were pre-incubated for 1 h with a commercial rabbit polyclonal anti-TRPC1 antibody (15 μg/ml, Alomone Labs, Israel), which was designed to bind to amino acid residues 557–571 in the proposed pore forming region of TRPC1 and block the functioning of the channel. The aspecific antibody was a rabbit polyclonal a-dog IgG.

In experiments in which Ba^{2+} influx was measured, the data are reported as the 340/380 ratio, 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 or coverslip trace.

Table 1
Differential expression of TRPC subtype between proliferating and differentiated aNPCs.

Subtypes	Differential expression ratio of mRNA	TaqMan assay
TRPC1	12.65	Mm00441975_m1
TRPC2	–	Mm00441984_m1
TRPC3	0.12	Mm00444690_m1
TRPC4	1.82	Mm00444284_m1
TRPC5	0.03	Mm00437183_m1
TRPC6	0.06	Mm01176083_m1
TRPC7	0.81	Mm00442606_m1

2.3. In vitro cell proliferation assay

For the cell proliferation assay, neurospheres at passages 4–8 were dissociated and plated at a density of 1×10^5 cells/ml in 96-well plates. The cell proliferation rate was determined using the BrdU incorporation assay with a labeling and detection kit (Roche Diagnostics) in accordance with the instructions from the manufacturer. Briefly, BrdU (final concentration of 10 μM) was added, and the cells were incubated for 24 h. The cells were fixed and incubated with anti-BrdU peroxidase (20 U/ml) for 30 min at 37 °C. The color was developed by the addition of the peroxidase substrate 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), and the absorbance at 405 nm was measured (reference of 490 nm). In a subset of the wells, 100 μM 2-APB, 1 μM Gd^{3+}, Ruthenium Red, SKF96365, a blocking antibody of TRPC1 or aspecific antibody was added as above.

2.4. siRNA

All siRNA duplexes were obtained from Dharmacon. Cells were transfected 24 h after plating with siRNAs using the Lipofectamine RNAi-MAX (Invitrogen). Specific silencing was confirmed 48 h post-transfection by quantitative real-time RT-PCR and Western blotting as described below. Proliferation assays and calcium measurement experiments were only carried out when greater than 80% knockdown of the target was achieved relative to the nontargeting siRNA control.

2.5. Real-time RT-PCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen). All of the primers and probes were purchased from Applied Biosystems. Assays included mouse TRPC1–7 (Table 1), Orai1 (Mm00774349_m1) and STIM1 (Mm00486423_m1). Real-time PCR was performed as previously described [14]. The PCR products were visualized after electrophoresis using 3% agarose gels.

2.6. Western blotting

Western blotting was performed according to the published methods [17]. Briefly, lysates from aNPCs were sonicated for 10 s