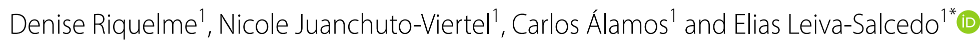
Molecular Brain



MICRO REPORT **Open Access**

TRPM4 inhibition slows neuritogenesis progression of cortical neurons

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Abstract
TRPM4 is a non-selective cation channel activated by intracellular Ca^{2+} but only permeable to monovalent cations, its activation regulates membrane potential and intracellular calcium. This channel participates in the migration and adhesion of non-excitable cells and forms an integral part of the focal adhesion complex. In neurons, TRPM4 expression starts before birth and its function at this stage is not clear, but it may function in processes such as neurite development. Here we investigate the role of TRPM4 in neuritogenesis. We found that neurons at DIV 0 express TRPM4, the inhibition of TRPM4 using 9-Ph reduces neurite number and slows the progression of neurite development, keeping neurons in stage 1. The genetic suppression of TRPM4 using an shRNA at later stages (DIV2) reduces neurite length. Conversely, at DIV 0, TRPM4 inhibition augments the Cch-induced $Ca^{2+}{}_i$ increase, altering the calcium homeostasis. Together, these results show that TRPM4 participates in progression of neurite development and suggest a critical role of the calcium modulation during this stage of neuronal development.

Keywords TRPM4, Neuritogenesis, Cortical neuron development, Intracellular calcium

Introduction

Neuritogenesis is the process by which neurons undergo morphological changes leading to neurite formation, allowing them to acquire a polarized morphology and establish somatic, dendritic, and axonal compartments [1]. This is the first step in neuronal development, particularly in excitatory cortical neurons, occurring alongside their migration from the ventricular zone to cortical areas [2].

Neurite formation begins with F-actin rearrangement, leading to membrane protrusion and lamellipodia and filopodia formation. During engorgement, microtubules advance into the lamellipodia and filopodia, leading to cylindrical neurite consolidation [3]. This process is highly regulated by intracellular Ca^{2+} ($Ca^{2+}{}_i$) [4, 5]; its absence arrests filopodia formation and neurite progression [6]. Inhibitors of $Ca^{2+}{}_i$ release or inhibitors of voltage gated calcium channels reduce neurite outgrowth [7], and complete suppression of $Ca^{2+}{}_i$ causes growth cone loss and cessation of neuritogenesis in cerebellar neurons [8]. Thus, ion channels and transporters affecting the resting membrane potential and intracellular calcium also regulate neurite elongation and neuronal development [9].

Calcium signals and membrane potential regulation are critical for neuritogenesis; changes in both can reduce migration and alter neurite number and development. TRPM4, a non-selective cation channel activated by $Ca^{2+}{}_i$ and permeable to monovalent cations, modulates calcium levels by controlling the Ca^{2+} driving force [10], or indirectly by activating voltage-gated calcium channels through depolarization [11]. TRPM4 regulates focal adhesion by modulating $Ca^{2+}{}_i$ in non-excitable cells, localizing in focal adhesions, with its trafficking regulated

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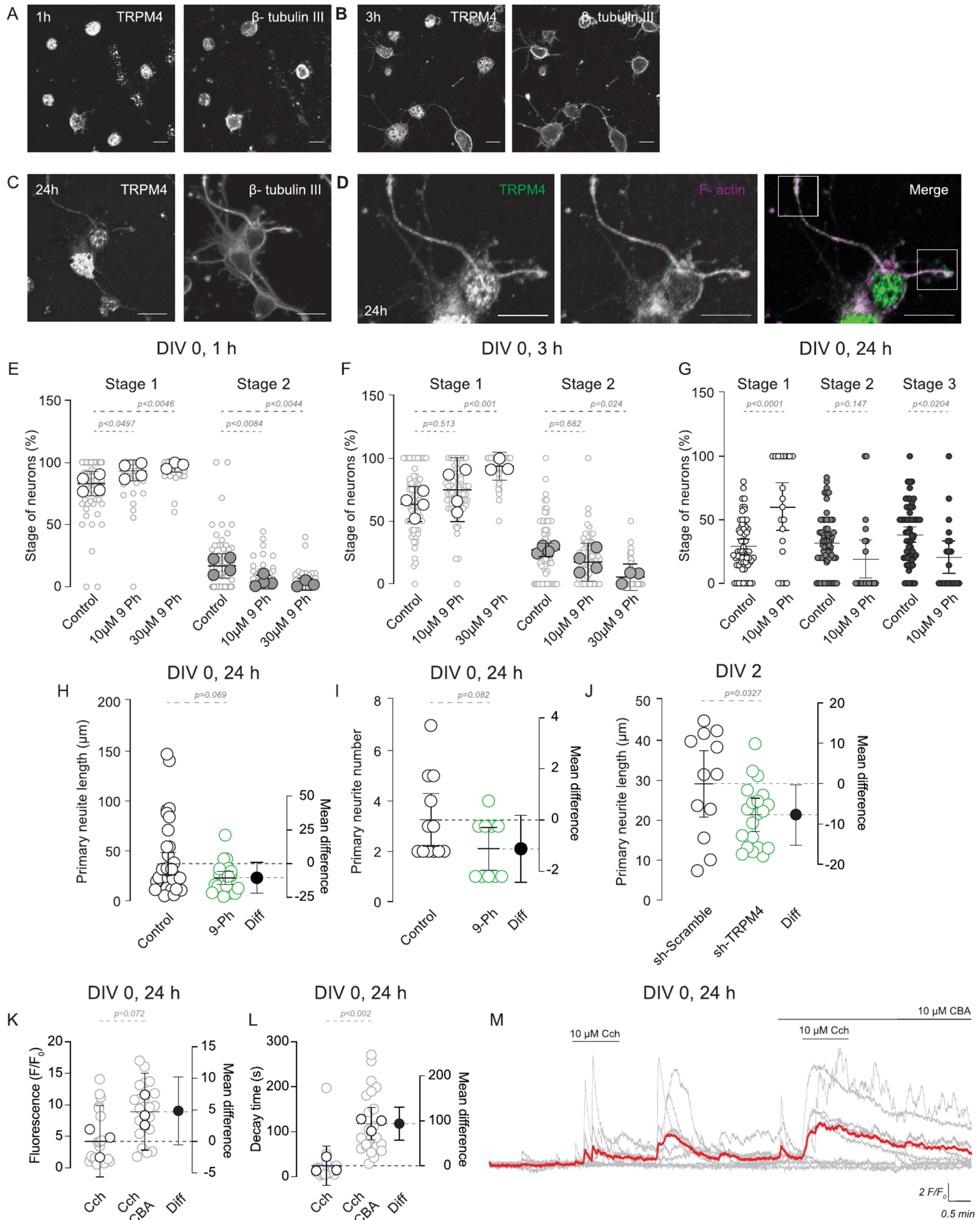

Fig. 1 (See legend on next page.)

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Fig. 1 TRPM4 inhibition reduces neurite development progression of cortical neurons. In (**A**) Immunostaining with TRPM4 and β-tubulin III at 1 h and (**B**) 3 h and (**C**) 24 h post disaggregation. (**D**) Zoom of the (**C**) showing the Immunostaining of TRPM4 and F-actin at 24 h, the white box indicates the expression in the growth cone. Scale bar = 10 μm. (**E**) Summary graph showing the effect of TRPM4 inhibition with 10 μM 9-Ph and 30 μM 9-Ph in the percentage of neurons in stage 1 and 2 of the neuritogenesis after 1 h (*N* = 4). (**F**) Summary graph showing the effect of TRPM4 inhibition with 10 μM 9-Ph and 30 μM 9-Ph in the percentage of neurons in stage 1 and 2 of the neuritogenesis after 3 h (*N* = 4). (**G**) Summary graph showing the effect of TRPM4 inhibition with 10 μM 9-Ph in the percentage of neurons in stage 1, 2 and 3 of the neuritogenesis after 24 h post culture (*N* = 2). Summary graph showing the length (**H**) and number (**I**) of the primary neurite before and after 10 μM 9-Ph. (**J**) Shows the primary neurite length in neurons transfected with shRNA-TRPM4 and the scramble sequence. Summary graph showing the quantification of the (**K**) maximum fluorescence and the (**L**) decay time of the signal induced by 10 μM Cch and 10 μM Cch + 10 μM CBA (*N* = 3). (**M**) Representative graph showing the $Ca^{2+}{}_i$ response, gray lines showing all neurons recorded, red line shows the average of the signal. Data is shown as the mean ± 95 C.I. and the right plot shows the mean difference, the *p*-value is shown above the graph

by microtubule-tracking protein EB, participating in focal adhesion disassembly and migration [12, 13]. Studies indicate TRPM4 is critical for adhesion and migration of various non-neuronal cells, such as mastocytes, lymphocytes, and melanoma cell lines. TRPM4 is expressed in the prefrontal cortex at least from P0 [14], suggesting a potential role in development. However, its specific role in neuritogenesis has not been explored. This study assesses TRPM4 role in neuritogenesis and $Ca^{2+}{}_i$ dynamics during early neurite development.

Results

To determine TRPM4 role in neuritogenesis, we assessed its expression in neurons during early development in culture. We performed immunostaining for TRPM4 and β-tubulin III in cortical neuron cultures at 1, 3, and 24 h after plating. TRPM4 was already expressed in neurons at 1 h after plating, with homogeneous expression in the soma. This pattern changed to somatic and neuritic localization at 3 h and became more pronounced at 24 h (Fig. 1A, B, and C).

To determine whether TRPM4 is coexpressed with F-actin at focal adhesions, a critical site for neurite outgrowth, we immunostained TRPM4 and used Phalloidin to detect F-actin. TRPM4 was coexpressed with F-actin, particularly in neurite growth cones (Fig. 1D), suggesting a role in neuritogenesis.

To examine the effect of TRPM4 inhibition on neuritogenesis, we used the model of polarity establishment with five stages: Stage 1, where spherical neurons start to extend filopodia and lamellipodia; Stage 2, where neurons present minor neurites; Stage 3, where one neurite grows to become an axon; and Stages 4 and 5, where neurons develop dendrites and form synapses, respectively [15]. We inhibited TRPM4 with 9-Phenanthrol (10 and 30 μM) in cortical neurons right after plating. After 1, 3, or 24 h of treatment, we fixed the neurons, immunolabeled with β-tubulin III and Phalloidin, then imaged neuronal morphology using a confocal microscope, and classified the neurons in the neuritogenesis stages (Suppl. Methods).

We found that 1-hour 9-Ph treatment slows the progression to Stage 2 (Control = 16.9%, 10 μM 9-Ph = 4.1%, 30 μM 9-Ph = 2.1%, Fig. 1E) and increases the percentage of cells in Stage 1 (Control = 83.1%, 10 μM 9-Ph = 93.4%, 30 μM 9-Ph = 97.8%, Fig. 1E). After 3 h of 9-Ph treatment, the percentage of neurons in Stage 2 is still reduced (Control = 27.7%, 10 μM 9-Ph = 17.9%, 30 μM 9-Ph = 5.7%, Fig. 1F), but there is an increase in neurons in Stage 1 (Control = 64.1%, 10 μM 9-Ph = 75.3%, 30 μM 9-Ph = 94.3%, Fig. 1F). After 24 h of treatment, we found that TRPM4 inhibition with 9-Ph slows neuritogenesis (Stage 1 Control = 31.2%, 10 μM 9-Ph = 60.1%, Fig. 1G), however, when we measure cell viability, we found that 24 h 9-Ph treatment induces cell death and decreases the number of the attached neurons (Suppl. Figure 1A). This suggest that the long-term treatment with 9-Ph affect neuronal adhesion and promote neuronal death.

Next, we treated neurons for 24 h with 10 μM 9-Ph and measured the primary neurites' length and number. We found that 9-Ph decreases the length (Control = 37.3 μm, 9-Ph = 23.1 μm, Fig. 1H) and number (Control = 3.2, 9-Ph = 2.1, Fig. 1I) of primary neurites. To corroborate this, we transfected neurons with shRNA against TRPM4 and measured primary neurite length at DIV2, this procedure reduces TRPM4 expression in 54.3% (Suppl. Figure 1B). We found that TRPM4 silencing decreases neurite length (shScramble = 29.3 μm, shTRPM4 = 21.1 μm, Fig. 1J). We also tested the $Ca^{2+}{}_i$ response and found that TRPM4 inhibition with CBA increases decay time (Cch = 24.5 s, Cch + CBA = 118.2 s, Fig. 1L and M), thus increasing the total $Ca^{2+}{}_i$ mobilized.

Discussion

TRPM4 is involved in non-neuronal cell migration, forming part of focal adhesions and regulating cell movement. Gradients of $Ca^{2+}{}_i$ are critical to promote neuritogenesis [6, 16], particularly low levels of $Ca^{2+}{}_i$ promote neurite outgrowth [6], in this context, our experiments shows that TRPM4 inhibition increases $Ca^{2+}{}_i$, thus may produce an opposite effect, and altering the optimal level of $Ca^{2+}{}_i$ necessary for neuritogenesis. This $Ca^{2+}{}_i$ increase may be due to the membrane potential of the neuron, which at this early stage is more depolarized and less responsive, since TRPM4 is expressed at least from DIV0 [14], TRPM4 could play critical roles in controlling depolarization and the $Ca^{2+}{}_i$ influx through VGCC during initial stages of development and impacting neurite outgrowth.

TRPM4 is expressed in lamellipodia where the presence of F-actin is critical for cell migration, our results shows that TRPM4 is coexpressed in areas rich in F-actin in the growth cone, thus in this area TRPM4 may participate in the tight control of the $Ca^{2+}{}_{i}$ which is critical for neurite outgrowth [6]. In non-excitable cells, TRPM4 participates in the focal adhesion turnover by modulating EB proteins, reducing focal adhesion disassembly, and decreasing lamellipodia extension [12, 13]; a similar effect may be playing TRPM4 in neurite outgrowth. We found that inhibiting TRPM4 with 9-Ph at the beginning of neuritogenesis is relevant for neurite stage progression. However, after 24 h inhibition, we observed no significant change in neurite length or number. Interestingly, TRPM4 silencing for 48 h significantly reduce neurite length, suggesting that TRPM4 also participates in later stage of neuritogenesis. In this context the use of strategies to silence the channel at earlier stages are necessary to complement our pharmacological approach and to fully investigate the effects of TRPM4 on the initial developmental stages of neuritogenesis. In conclusion, our findings suggest that TRPM4 plays a significant role in neuritogenesis by modulating intracellular calcium.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13041-024-01140-3.

Supplementary Material 1: Supplementary Methods

Supplementary Material 2: Supplementary Figure 1

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Author contributions

D.R. and E. L-S. conceived the study. D.R., C.A., N. J. and E. L-S. performed the experiments and analyzed the data. D.R. and E. L-S. wrote the manuscript, and all authors edited the manuscript.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

All animal experiments were performed following approval from the Ethic committee of the University of Santiago of Chile.

Consent for publication

All authors consent to publication.

Competing interests

The authors declare no competing interests.

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