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Hypoxia-Induced Neurite Outgrowth Involves Regulation Through TRPM7

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Abstract

Transient receptor potential melastatin 7 (TRPM7) is a ubiquitously expressed divalent cation channel that plays a key role in cell functions such as ion homeostasis, cell proliferation, survival, and cytoskeletal dynamics and mediates cells death in hypoxic and ischemic conditions. Previously, TRPM7 was found to play a role in the neurite outgrowth and maturation of primary hippocampal neurons. Either knockdown of TRPM7 with target-specific shRNA or blocking channel conductance by a specific blocker waixenicin A enhanced axonal outgrowth in the primary neuronal culture. In this study, we investigated whether and how TPRM7 is involved in hypoxia-altered neurite outgrowth patterns in E16 hippocampal neuron cultures. We demonstrate that short-term hypoxia activated the MEK/ERK and PI3K/Akt pathways, reduced TRPM7 activity, and enhanced axonal outgrowth of neuronal cultures. On the other hand, long-term hypoxia caused a progressive retraction of axons and dendrites that could be attenuated by the TRPM7-specific inhibitor waixenicin A. Further, we demonstrate that in the presence of astrocytes, axonal retraction in long-term hypoxic conditions was enhanced, and TRPM7 block by waixenicin A prevented this retraction. Our data demonstrate the effect of hypoxia on TRPM7 activity and axonal outgrowth/retraction in cultures with or without astrocytes present.

Keywords TRPM7 · Ion channels · Waixenicin A · Neurite · Outgrowth · Hypoxia

Introduction

The transient receptor potential (TRP) superfamily of ion channels is widely expressed in various cell and tissue types and plays a role in many different physiological processes. The TRP superfamily is divided into six families based on sequence homology: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), TRPA (ankyrin). The first discovery of the TRP superfamily began with Cosens and Manning in 1969, who discovered a phenotype of drosophila that exhibited a transient response to light [1]. Since then, 28 cation permeable channels have been discovered within the TRP superfamily. In company with their widespread expression, the TRP channels comprise a diverse range of functions in physiological and pathophysiological contexts.

Transient receptor potential melastatin 7 (TRPM7) is a ubiquitously expressed divalent cation channel permeable to Ca^{2+}, Zn^{2+}, and Mg^{2+} [2, 3]. Similar to other TRP channels, TRPM7 shares the characteristic six transmembrane domains with a cytosolic amino (n-) and carboxyl (c-)

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terminus; however, it differs in its c-terminal having an atypical α-kinase domain, allowing the channel to function as a chanzyme [4]. The α-kinase primarily phosphorylates serine and threonine residues and has a variety of downstream targets. TRPM7 has been implicated in a diverse spectrum of pathologies such as ischemic diseases in both the heart and brain [5], cancer [6, 7], neurodegeneration [8], and hypertension [9]. TRPM7's physiological roles include cell proliferation [10], cytoskeleton regulation [11], ion homeostasis [12], embryological [13, 14] development, and neurite outgrowth [15]. With many of these roles, Ca^{2+} conductivity underlies the mechanism of TRPM7's involvement.

Neurite outgrowth is a key process in neuronal development as well as regeneration following injury and is dependent on a complex interface of dynamics and mechanics within the cytoskeleton. The growth cone is the motile structure at the ends of dendrites and axons and is dependent on cytoskeletal proteins actin filaments and microtubules [16]. There are three functionally distinct regions within the growth cone, the peripheral domain, the transition zone, and the central domain [17]. Within the peripheral domain and the transition zone, F-actin is the most critical cytoskeletal structure, where constant turnover is a fundamental component in growth cone motility forward. As TRPM7 is calcium permeable and plays a role in cytoskeleton regulation, we previously were able to show that not only is TRPM7 highly expressed at the tips of the growth cone, but also that TRPM7 regulates neurite outgrowth, dependent on calcium via its regulation of F-actin and α-actinin-1 [15]. Both the genetic knockdown of TRPM7 and the pharmacological blockade with the specific inhibitor waixenicin A enhanced axonal outgrowth. In the case of waixenicin A, both TRPM7-like current suppression and axonal outgrowth were similarly dose-dependent in the nano-molar range [15].

While hypoxic brain injury is often the consequence of ischemia or reduced blood supply to the brain, hypoxic injury can often occur without ischemia when arterial oxygen levels are reduced. In adults and children, this type of hypoxia can occur during severe anemia, drowning, cyanide poisoning, or at high altitudes, and prolonged hypoxia can be damaging to the brain, causing severe and irreversible neurocognitive deficits [18–21]. In a fetus, intrauterine hypoxia can occur due to a variety of maternal, placental, and fetal conditions, some of which are a hypoxic environment (high altitudes), maternal infections, hematological conditions such as thalassemia (reduced hemoglobin production due to genetic mutation) or iron deficiency anemia, chronic inflammation, and smoking [22]. All these conditions limit maternal oxygen uptake, thus reducing oxygen supply to the fetus in the presence of adequate blood flow [22]. The effects of reduced oxygen conditions on neurite outgrowth and development are still under debate, and different studies report conflicting findings [23–25]. TRPM7 activity has been shown to be potentiated by a variety of extracellular conditions that may occur due to hypoxia, such as changes in pH [26], production of ROS [27], and changes in extracellular divalent cations [28]. This evidence, taken together with previous findings that TRPM7 activity may negatively regulate axonal outgrowth and development, suggests that TRPM7 activity may also play a role in axonal outgrowth under hypoxic conditions. Our study characterizes the effect of hypoxia on TRPM7 activity and axonal outgrowth/retraction with and without astrocytes present in culture.

Methods

Cell Culture

Dissociated culture of primary embryonic hippocampal neurons: All procedures were performed in accordance with animal welfare guidelines at the University of Toronto and were approved by the institutional animal care and use committee. Embryonic hippocampal cultures were prepared from E16.5 CD1 mice as described previously [29]. Dissected hippocampi were digested with 0.025% Trypsin/EDTA at 37 °C for 15 min. Cell density was determined using an Improved Neubauer hemocytometer, and 1.0×10^4 cells were plated on poly-D-lysine (Sigma)-coated glass coverslips (12 mm #1 German Glass, Bellco cat #1943–10,012). The cells were kept in 5% CO_2 at 37 °C in culture medium.

Drug Treatment of Hippocampal Neurons

Waixenicin A was obtained through collaboration with Dr. Andrea Fleig (The Queen's Medical Center) and Dr. F. David Horgen (Hawaii Pacific University) [10]. The stock of waixenicin A was prepared by dissolving the compound in 100% methanol and adding PBS to dilute methanol to 2.5% concentration. Waixenicin A was added to cell cultures on the day in vitro (DIV) 2, 24 h post-plating. Naltriben mesylate (Tocris, #0892) was dissolved in DMSO and added to cultures on DIV4. Control neurons were either left untreated or treated with the appropriate vehicle at the concentration shown below (Table 1).

Table 1 Drug concentrations for the treatment of hippocampal cultures

Compound	Final concentration in culture
Waixenicin A (drug)	500 nM
Methanol (vehicle)	0.0025% (vol/vol)
Naltriben mesylate (drug)	500 nM, 1 µM, 10 µM, 25 µM, 50 µM
DMSO (vehicle)	0.001% (vol/vol)

Hypoxic Treatment of Hippocampal Neurons

Neuronal cultures and neuron-astrocyte co-cultures were grown under normal conditions (37 °C, 5% CO_2) until DIV4. On DIV4, the cultures were transferred into an air-tight Hypoxia Incubator Chamber, which was flushed with hypoxic gas (5% O_2) for 5 min as described previously [24]. The Hypoxia Incubator Chamber was then transferred into an incubator (37 °C) and kept there for up to 10 h. The chamber was flushed with hypoxic gas every 2 h. Upon completion of hypoxic treatment, cells were either immediately fixed with 4% paraformaldehyde (PFA) and prepared for immunolabeling or transferred to an incubator with normal conditions.

Electrophysiology

Whole-cell patch-clamp recording was used to study TRPM7-like currents in primary mouse neurons using an Axopatch 700B (Axon Instruments, Inc.). Before patching, neurons were either in normal conditions (normoxia; control) or exposed to short-term hypoxia (~ 1 h) or long-term hypoxia (> 2.5 h) in a hypoxic chamber. Currents were recorded using a 400 ms voltage ramp protocol (− 100 to + 100 mV) with an interval of 5 s at 2 kHz and digitized at 5 kHz. pClamp 9.2 software was used for data acquisition, and Clampfit 9.2 was used for data analysis. All experiments were carried out at room temperature. Patch pipette resistance was between 5 and 9 megaohms after filling with pipette solution containing (in mM): 145 CsMSF, 8 NaCl, 0.5 $MgCl_2$, 10 EGTA, and 10 HEPES, pH adjusted to 7.2 with CsOH. The bath solution contained (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 20 HEPES, and 10 glucose (pH adjusted to 7.4 with NaOH).

Immunocytochemistry

The immunocytochemistry(ICC) has been described previously [15]. In brief, the cultured neuronal cells were fixed with 4% paraformaldehyde and 4 % sucrose in PBS for 15 min. Afterward, the fixed cells were permeabilizedwith 0.1 % Triton X-100 in PBS for 10 min and blocked with ablocker solution (containing 2 %bovine serum albumin, 2 % fetal bovine serum, and 0.2 % fish gelatin in PBS) for 1 h (at roomtemperature). The cells were then incubated with the primary antibodies todifferentiate between axons anddendrites using mouse monoclonal anti-tau-1 (axonal marker; 1:500, Millipore, MAB3420) and chickenpolyclonal anti-microtubule associated protein 2(MAP2) (dendritic marker; 1:500, Millipore, Ab15452).

Image Acquisition for Neurite Length Analysis

Images of neurons were captured with either a Carl Zeiss Confocal Laser Scanning Microscope LSM700 with either 63 × DIC (NA 1.40) or 40 × DIC (NA 1.3) oil immersion lenses and 488-, and 543-nm lasers [15]. Each Z-plane was 0.3 µm. All cells were imaged using the same magnification and laser settings. All cells were imaged at a resolution of 1024 × 1024 pixels using the same magnification and laser settings.

Neurite Length Analysis

Neurite lengths were analyzed with SynD, a semi-automated image analysis routine [30]. For neurons (DIV > 6) that require a larger field of view to capture a single neuron, multiple images were taken under the 40 × lens first, and mosaics of overlapping images were assembled in ImageJ (NIH, http://rsb.info.nih.gov/ij) using MosaicJ plugin before neurite length analysis.

Western Blotting

The protein was collected from dissociated mouse hippocampal neuronal cells, MDA-MB-213 cells, or ipsilateral hemispheres of pups 6 or 24 h after HI in RIPA buffer (Santa Cruz Biotechnology, sc-24948) with protease inhibitor mixture (Santa Cruz Biotechnology, sc-29131). The protein samples (20 µg/well) were separated on a 10% SDS-PAGE gel, and proteins were then transferred to a nitrocellulose membrane (200 mA per gel, 60 min). Membranes were incubated with specific primary antibodies summarized in Table 2 at 4 °C overnight. Horseradish peroxidase conjugated anti-mouse (1:7500; Chemicon AP130P), anti-rabbit (1:7500; Chemicon AP132S), and anti-goat (1:7500; Chemicon AP180P) IgG antibodies were used as secondary antibodies and were detected with the ECL system (PerkinElmer, Inc., USA). Images were analyzed using an image-analysis system (NIH Image J 1.47v).

Statistical Analysis

Data are presented as the mean ± SEM. Statistical analysis was performed using GraphPad Prism (GraphPad Software,

Table 2 Media/solution composition for HEK-293 cell culture

Solution	Ingredients	Concentration
Coating solution	Poly-D-Lysine in sterile H_2O	
Growth medium	Minimum essential medium (MEM)	
	Fetal bovine serum	10% (vol/vol)
	GlutaMAX supplement	1% (vol/vol)
	Zeocin	0.2% (vol/vol)
	Blasticidin	0.1% (vol/vol)
	Penicillin–streptomycin	1% (vol/vol)
Induction	Tetracycline	1 mg/mL

San Diego, CA). The multiple comparisons of means between experimental and control conditions were performed with Bonferroni post hoc test following the one-way ANOVA. Values of $p < 0.05$ are taken as statistically significant.

Results

Short-Term Hypoxia Enhances Axonal Outgrowth

It was found that a 1-h-long hypoxia treatment significantly enhanced axonal lengths in all treatment groups compared to neurons kept under normoxic conditions (pre-hypoxia control: 253 ± 19 μm, $n = 43$; normoxia untreated: 241 ± 22 μm, $n = 43$; normoxia vehicle: 216 ± 13 μm, $n = 68$; normoxia waixenicin A: 335 ± 20 μm, $n = 63$; hypoxia untreated: 304 ± 26 μm, $n = 32$; hypoxia vehicle: 300 ± 21 μm, $n = 50$; hypoxia waixenicin A: 338 ± 20 μm, $n = 53$) (Fig. 1A). Dendritic analysis revealed a significant reduction in length during hypoxia treatment, which could not be prevented by waixenicin A treatment (pre-hypoxia control: 200 ± 17 μm, $n = 41$; normoxia untreated: 162 ± 17 μm, $n = 43$; normoxia vehicle: 150 ± 12 μm, $n = 68$; normoxia waixenicin A: 171 ± 11 μm, $n = 63$; hypoxia untreated: 120 ± 13 μm, $n = 32$; hypoxia vehicle: 126 ± 8 μm, $n = 50$; hypoxia waixenicin A: 145 ± 10 μm, $n = 53$) (Fig. 1C). Representative images of the neurons in different treatment groups are shown in Fig. 1A.

Short-Term Hypoxia Causes Downregulation of TRPM7 Protein Levels and Activation of MEK/ERK and PI3K/Akt Signaling Pathways

Western blot results indicated a downregulation of TRPM7 expression together with activation of ERK and Akt signaling pathways (Fig. 2). These data suggest that short-term hypoxia causes an increase in axonal outgrowth through concurrent activation of pro-growth ERK and Akt signaling and downregulation of TRPM7 protein levels.

Long-Term Hypoxia Causes Axonal and Dendritic Retraction

We next characterized the effect of long-term hypoxia on axonal and dendritic outgrowth. Prolonged (2, 4, 6, 8, and 10 h) hypoxic treatment caused a significant reduction of both axonal (Fig. 3A) and dendritic lengths (Fig. 3B), compared to normoxic neurons. However, this retraction was attenuated in groups treated with waixenicin A, suggesting the involvement of TRPM7 in later stages of the hypoxic response.

Naltriben Exacerbates the Effect of Long-Term Hypoxia on Axonal Outgrowth

To confirm the involvement of TRPM7 activity in long-term hypoxia-induced axonal retraction, axonal lengths were measured in neurons subjected to hypoxia for 1, 2, and 4 h and used as controls. As seen in Fig. 4, naltriben causes axonal retraction in normoxic neurons and significantly exacerbates axonal retraction in hypoxic neurons after 2 and 4 h of hypoxia.

Short-Term Hypoxia Attenuates and Long-Term Hypoxia Potentiates TRPM7-Like Current in Hippocampal Neurons

To examine the effects that short and long-term hypoxia had on TRPM7 activity, whole-cell patch-clamp recordings were carried out on DIV4-5 primary hippocampal neurons in normoxic conditions (control), after short- (~ 1 h) or long-term (> 2 h) hypoxia. As a proof-of-principle assessment of whether waixenicin A (500 nM) can inhibit TRPM7-like activity in primary hippocampal neurons under hypoxic conditions, we used the waixenicin A-sensitive current component for all analyses. Note that since a pan-inhibitor cocktail (500 nM TTX, 25 μM APV, 40 μM CNQX, and 5 μM nimodipine) was not added, current contamination from voltage-gated sodium and calcium channels and AMPA receptors cannot be ruled out. Representative traces of TRPM7-like current in normoxic neurons, neurons after short-term hypoxia, and neurons after long-term hypoxia with and without application of waixenicin A are shown in Figs. 5A, B, and C, respectively. As shown in Fig. 5D, we found that short-term hypoxia reduced the TRPM7-like current density from 10.51 ± 1.16 to 6.55 ± 0.83 pA/pF ($p < 0.05$; $n = 4$/group). On the contrary, long-term hypoxia enhanced the TRPM7-like current density to 15.41 ± 0.61 pA/pF ($p < 0.05$; $n = 4$/group) (Fig. 5D). This finding shows that, even on the functional level, TRPM7-like activity is affected by the duration of hypoxic exposure, thus suggesting that short-term hypoxia can potentially be neuroprotective by reducing TRPM7-like activity. These results confirm our previous finding that on the functional level, TRPM7 activity is affected by the duration of hypoxic exposure.

Waixenicin A Attenuates Hypoxia-Induced Axonal Retraction When Administered Immediately After Hypoxia

To determine if waixenicin A application can rescue axonal retraction induced by long-term hypoxia, neurons were subjected to post-treatment paradigm. Briefly, neurons were grown for 4 days under normal conditions. On DIV4,

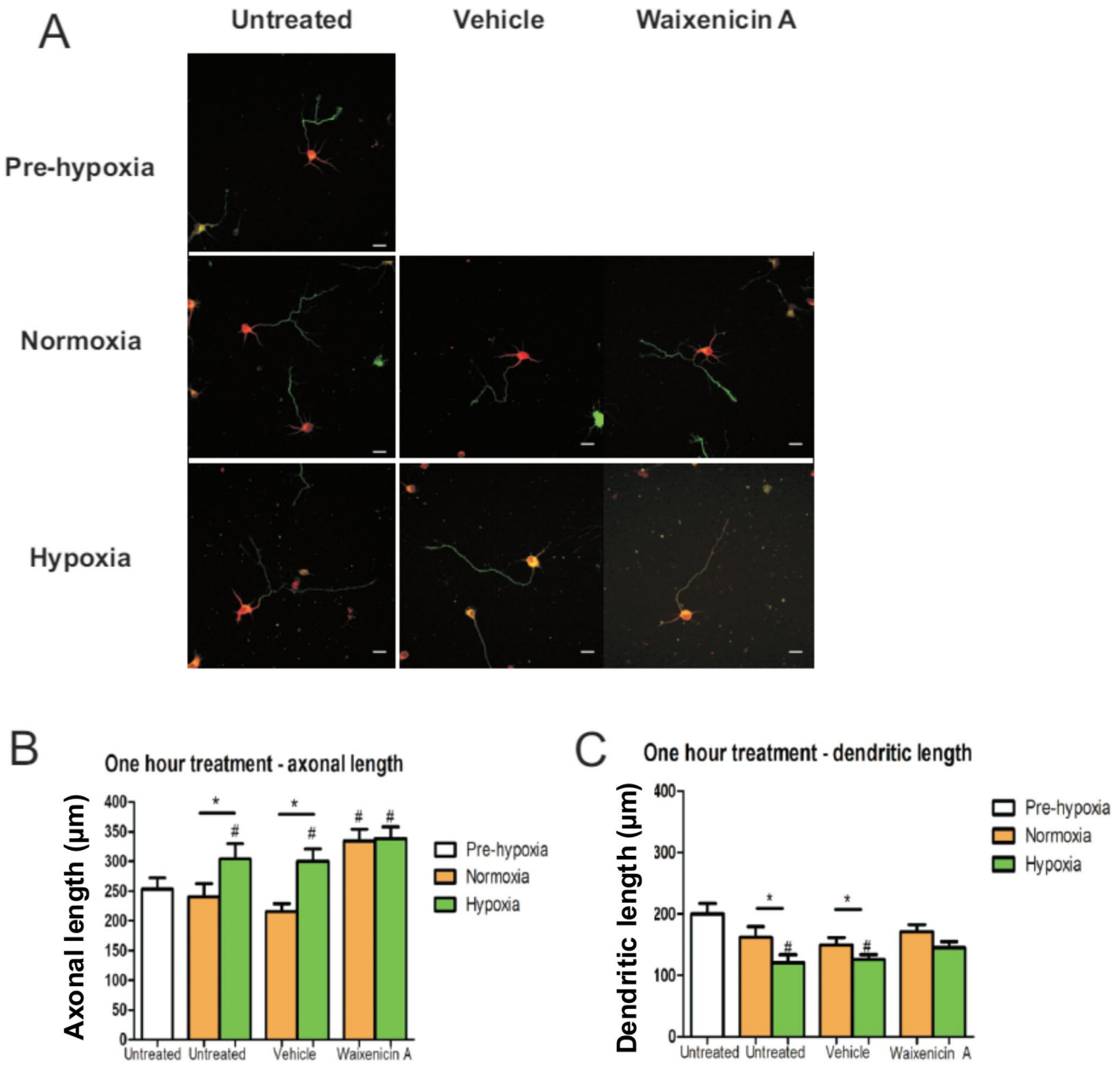

Fig. 1 Short-term hypoxia enhances axonal outgrowth. **A** Representative images of neurons in different treatments under normoxic and hypoxic conditions. **B** Mean axonal and **C** mean dendritic lengths of neurons in different treatment conditions. All data are presented as mean ± SEM, $*p < 0.05$ (one-way ANOVA with Bonferroni post hoc)

neurons were exposed to 2-h hypoxia. This treatment length was chosen because this is the shortest length of hypoxia that was shown to induce axonal retraction and potentiate TRPM7 current. Immediately after hypoxic treatment, neurons were treated with 500 nM waixenicin A, vehicle or left untreated, and were transferred to the incubator with normal conditions. After 24 h of recovery, neurons were fixed, and axonal and dendritic lengths were measured. Neurons that were kept under normoxic conditions were used as control. It was found that during the recovery period following 2-h hypoxia, neurons underwent further axonal retraction. This axonal retraction was rescued by waixenicin A application, suggesting that potentiation of TRPM7 activity by hypoxia could also persist during the recovery process and cause further axonal retraction under normal conditions (neurons immediately after hypoxia, untreated: 184.7 ± 11 µm, $n = 52$; neurons 24 h after hypoxia, untreated: 140.2 ± 11 µm, $n = 51$; vehicle: 130.4 ± 8.7 µm, $n = 41$; waixenicin A: 188.7 ± 9 µm, $n = 53$) (Fig. 6A). Dendritic lengths and the total number of neurites increased 24 h after hypoxia (Fig. 6B and C).

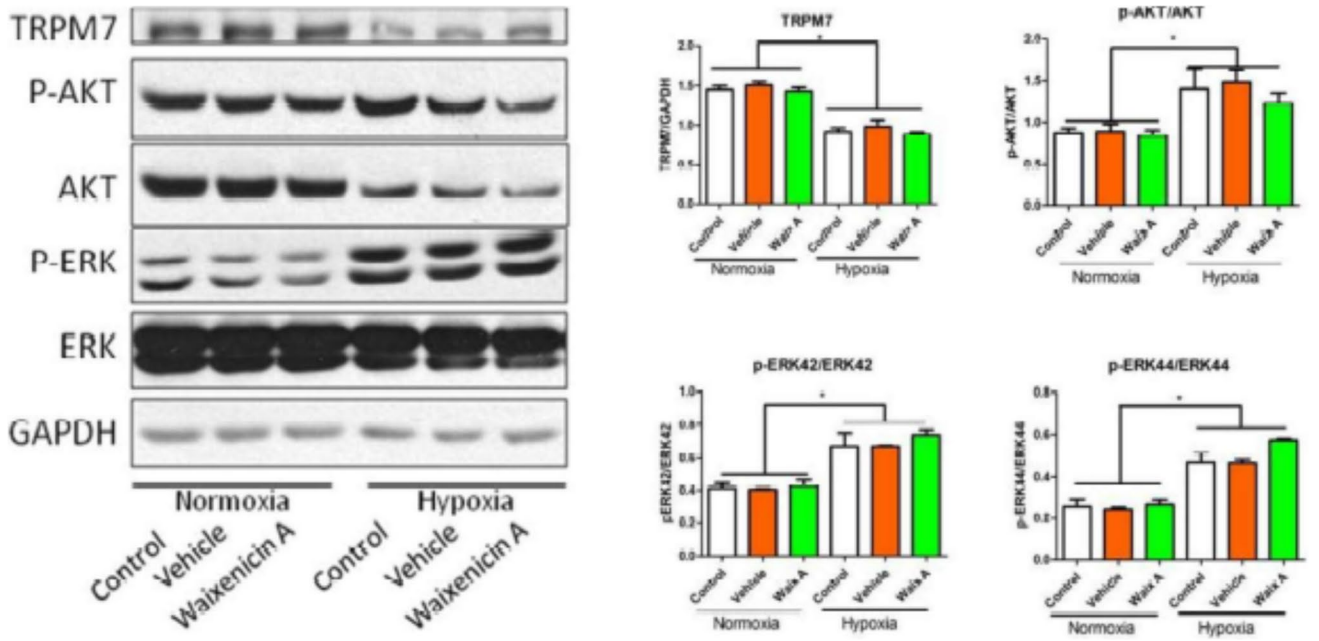

Fig. 2 Short-term hypoxia causes downregulation of TRPM7 and activation of ERK and Akt signaling pathways. Representative western blot of protein extracted from DIV4 hippocampal neurons immediately following 1-h hypoxia and quantification of TRPM7, p-Akt/Akt, and p-ERK/ERK protein levels in hippocampal neurons immediately following 1-h hypoxia ($N=3$). All data are presented as mean ± SEM, $^*p<0.05$ (one-way ANOVA with Bonferroni post hoc)

These findings demonstrated that axons continue to retract even during recovery after hypoxia and TRPM7 block by waixenicin A can attenuate this retraction. However, TRPM7 block does not promote further outgrowth in these neurons.

The Presence of Astrocytes in Culture Exacerbates Hypoxia-Induced Neurite Retraction

Next, we examined how the presence of astrocytes in the culture affected hypoxia-induced neurite retraction and TRPM7-like currents in neurons. Neuron-astrocyte co-cultures were grown for 4 days. On DIV4, the cultures were separated into three treatment groups: untreated, vehicle-treated, and waixenicin A-treated. Immediately after the treatment, cultures were subjected to hypoxia for 1, 2, 4, and 6 h and fixed immediately after. Axonal and dendritic lengths were analyzed and compared to neurons in the same treatment group that was kept under normoxic conditions. As seen in Fig. 7A, the presence of astrocytes in the culture abolished an increase in axonal outgrowth caused by short-term hypoxia in neuronal cultures. At the same time, the retraction of axons caused by long-term hypoxia was much faster in co-cultures when compared to neuronal cultures (neuronal cultures: 27% after 2 h, 51% after 4 h, 54% after 6 h; neuron-astrocyte co-cultures: 53% after 2 h, 76% after 4 h, 83% after 6 h). TRPM7 block by waixenicin A prevented this retraction for up to 4 h of hypoxia, suggesting that TRPM7 activity plays a role in axonal retraction in this culture system. Dendrites showed a retraction pattern similar to axons (Fig. 7B).

The Effect of Astrocytes on TRPM7-Like Current in Hippocampal Neurons

Next, the effect of astrocytes on TRPM7-like currents in neurons was investigated. As a proof-of-principle assessment of whether waixenicin A can inhibit TRPM7-like activity in primary hippocampal neurons under hypoxic conditions, we used the waixenicin A-sensitive current component for all analyses. Note that since a pan-inhibitor cocktail (500 nM TTX, 25 μM APV, 40 μM CNQX, and 5 μM nimodipine) was not added, current contamination from voltage-gated sodium and calcium channels and AMPA receptors cannot be ruled out. Representative traces of TRPM7-like current in normoxic neurons, neurons after short-term hypoxia, and neurons after long-term hypoxia with and without application of waixenicin A are shown in Fig. 8A, B, and C, respectively. In neurons that were co-cultured with glial cells, short-term hypoxia had no significant effect on neuronal TRPM7-like activity (i.e., 8.59 ± 0.81 pA/pF and 9.23 ± 1.61 pA/pF under normoxic control and short-term hypoxic conditions, respectively Fig. 8D). Nevertheless, long-term hypoxia increased neuronal TRPM7-like activity to 12.84 ± 0.80 pA/pF (Fig. 8D) ($p<0.05$ compared to normoxic control; not significant compared to short-term hypoxia group). These results

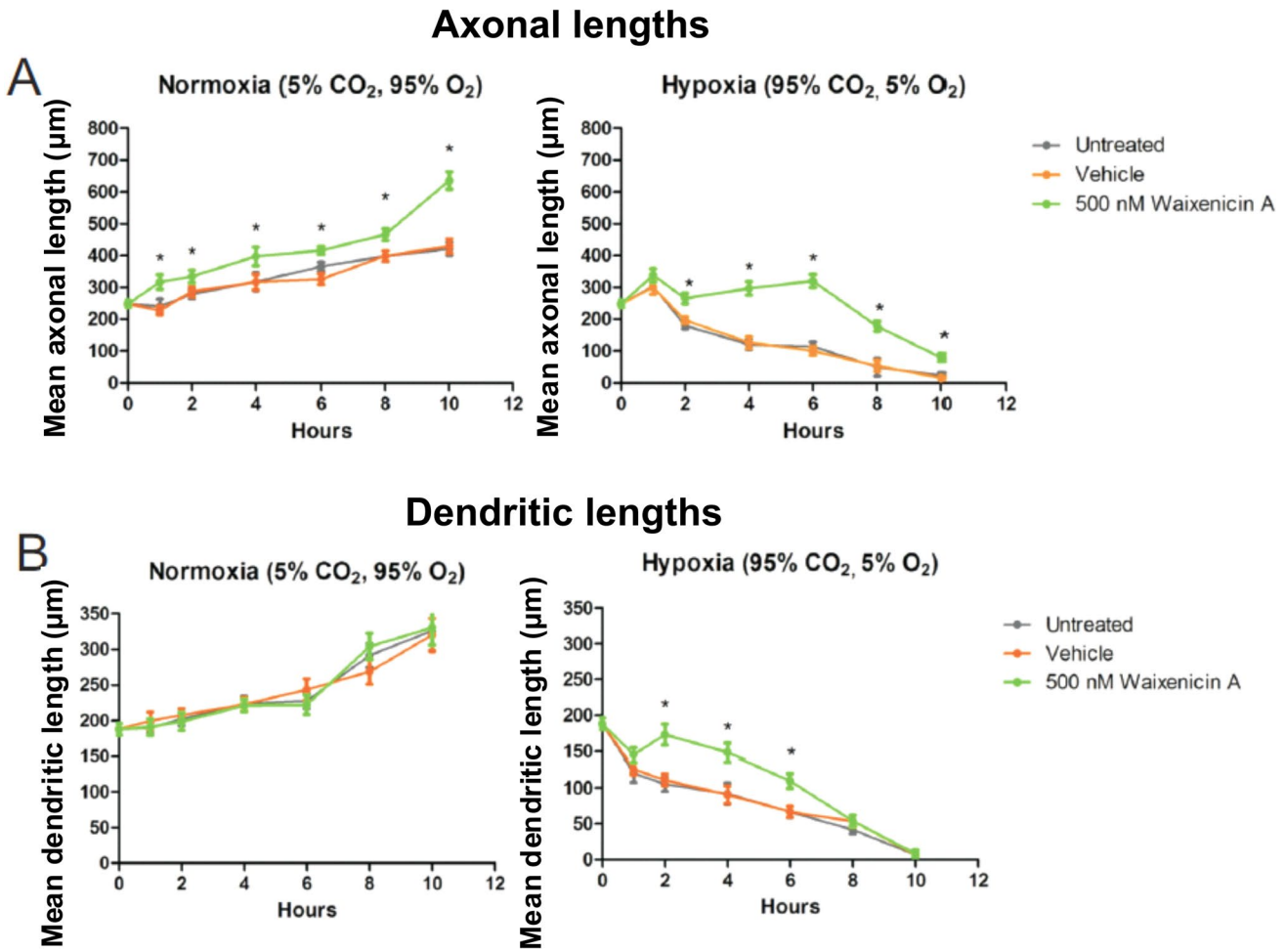

Fig. 3 Long-term hypoxia causes axonal and dendritic retraction and TRPM7 block attenuates this retraction. **A** Axonal and **B** dendritic lengths under normoxic and hypoxic conditions. All data are presented as mean ± SEM, $*p < 0.05$ (ANOVA with Bonferroni post hoc)

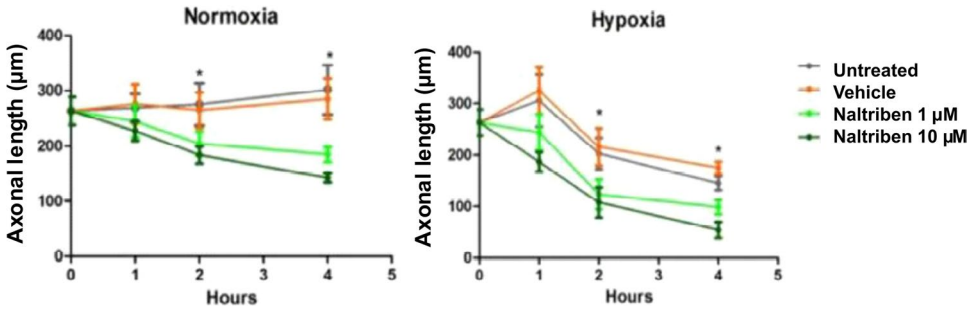

Fig. 4 Naltriben exacerbates axonal retraction under hypoxic conditions. Axonal length of DIV4 neurons lengths under normoxic and hypoxic conditions with and without the addition of naltriben. All data are presented as mean ± SEM, $*p < 0.05$ (ANOVA with Bonferroni post hoc).

demonstrate that in this co-culture system, TRPM7 was also regulated by hypoxic conditions.

The Effect of Soluble Factors on Axonal Retraction

Finally, we investigated whether the effect of astrocytes on axonal retraction was due to soluble factors released by these cells, cell–cell interactions, or both. We performed a set of media switch experiments outlined below. Neuronal cultures or neuron-astrocyte co-cultures were grown for 4 days under normal conditions. On DIV4, these cultures were subjected to hypoxia for 1, 2, 4, and 6 h. Immediately after, the media from these cultures were collected and placed on DIV4 neuronal cultures under normal conditions

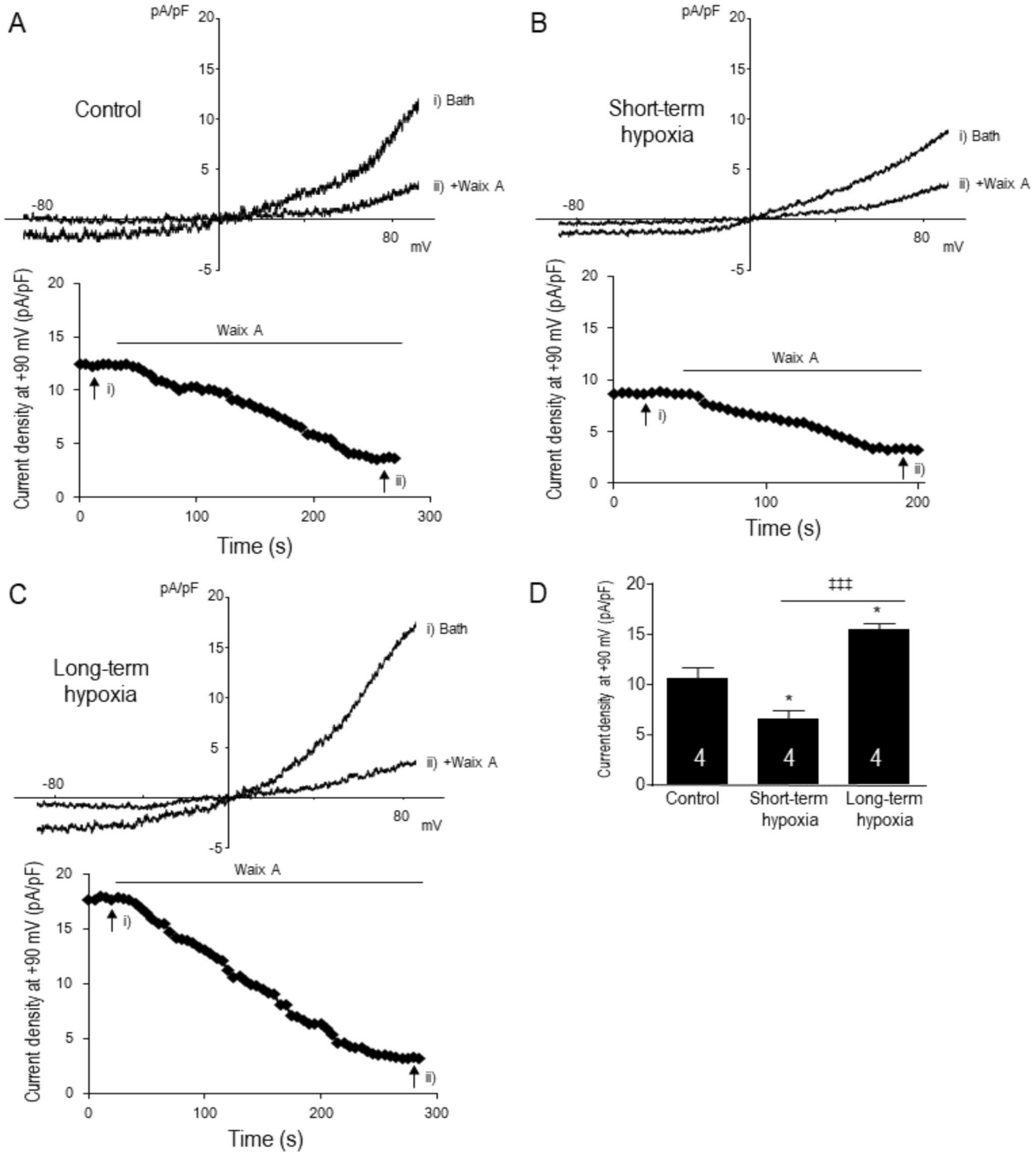

Fig. 5 TRPM7-like activity was reduced with short-term hypoxia and enhanced with long-term hypoxia. Representative traces and time course of TRPM7-like current density were assessed at + 90 mV, and before and after application of 500 nM waixenicin A in **A** control (normoxic) neurons; **B** short-term hypoxic (~ 1 h in hypoxic chamber) neurons; and **C** long-term hypoxic (> 2.5 h in hypoxic chamber) neurons. Arrows on the time course indicate corresponding time points shown in traces. **D** Comparison of TRPM7-like current density (i.e., waixenicin A-sensitive inhibition component) in control (normoxic), short-term hypoxic, and long-term hypoxic neurons. Asterisk represents $p < 0.05$, comparison to control; ‡‡‡ $p < 0.001$ (one-way ANOVA with Bonferroni multiple comparison tests; $n = 4$/group). Bars represent SEM

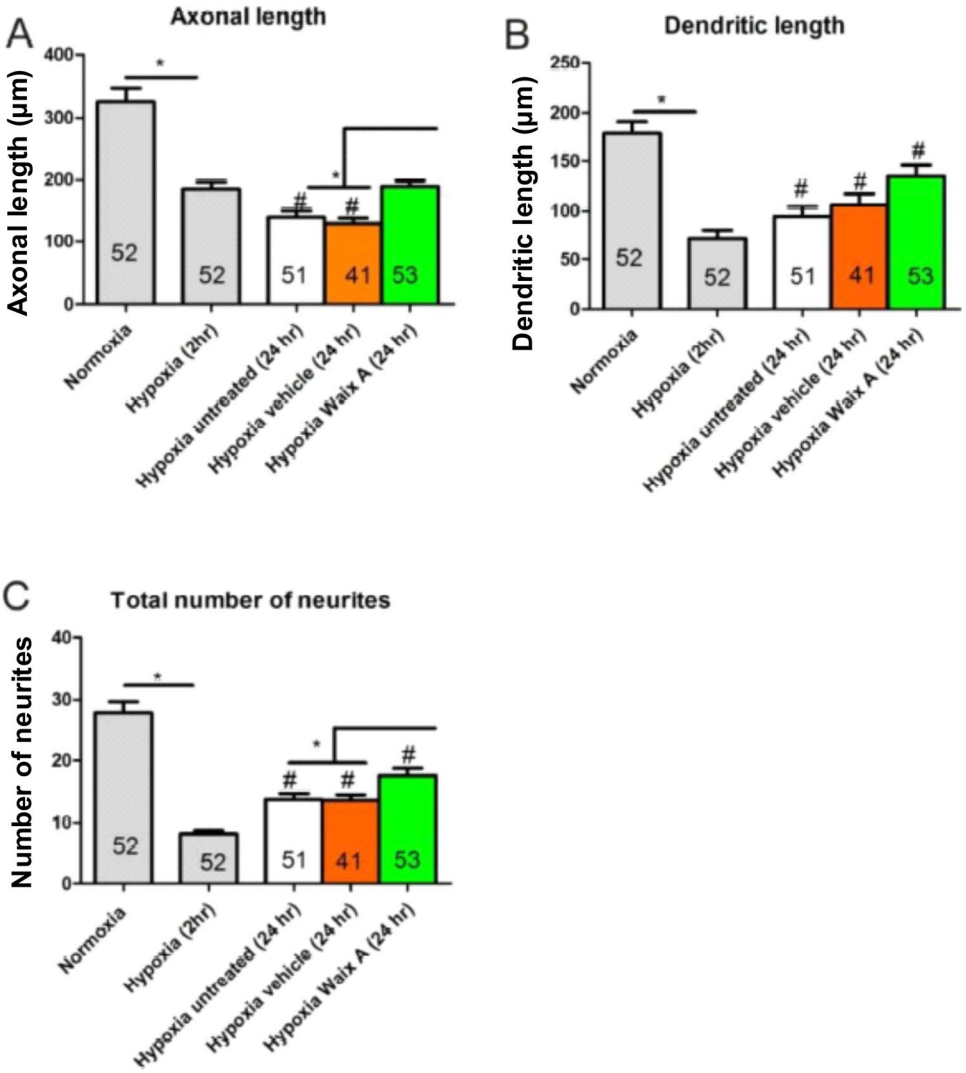

Fig. 6 TRPM7 block attenuates axonal retraction when administered immediately after hypoxia. **A** Axonal lengths, **B** dendritic lengths, and **C** total number of neurites of neurons before (normoxia), immediately after, and 24 h after hypoxia. All data are presented as mean ± SEM, $*p < 0.05$, and $\#p < 0.05$ compared to immediately after hypoxia (one-way ANOVA with Bonferroni post hoc)

for 2 h. After 2 h, neuronal cultures that received conditioned media were fixed with 4% PFA and prepared for immunolabeling. Control cultures were kept under normal conditions and fixed at 1-, 2-, 4-, and 6-h time points for comparison. At the time of the media switch, neurons receiving conditioned media were also treated with vehicle, waixenicin A, or left untreated. Overall, axonal lengths followed the patterns described in previous sections, suggesting that the soluble factors released under hypoxic conditions may play an important role in initiating axonal retraction. The media collected from hypoxic neuronal cultures resulted in an increase in axonal outgrowth after short-term hypoxia and a subsequent trend towards axonal retraction (Fig. 9A). The media collected from hypoxic neuron-astrocyte co-cultures resulted in progressive axonal retraction as hypoxia exposure increased (Fig. 9B).

Discussion

In this study, we demonstrate for the first time the effects of short- and long-term hypoxia on TRPM7-regulated neurite outgrowth, the suggested signaling pathways affected, and how the presence of astrocytes impacts the effects of hypoxia. Previous studies have demonstrated the role of TRPM7 in regulating neurite outgrowth and maturation as well as the expression of TRPM7 in the growth cone [15]. Overall, the findings suggest that short-term hypoxia enhances axonal outgrowth through downregulation of TRPM7 activity, while long-term hypoxia leads to axonal retraction at least in part due to potentiation of TRPM7 activity. This axonal retraction can be prevented by the application of waixenicin A prior to the onset of hypoxia. We also characterize the effect of hypoxia on TRPM7 activity and axonal outgrowth/

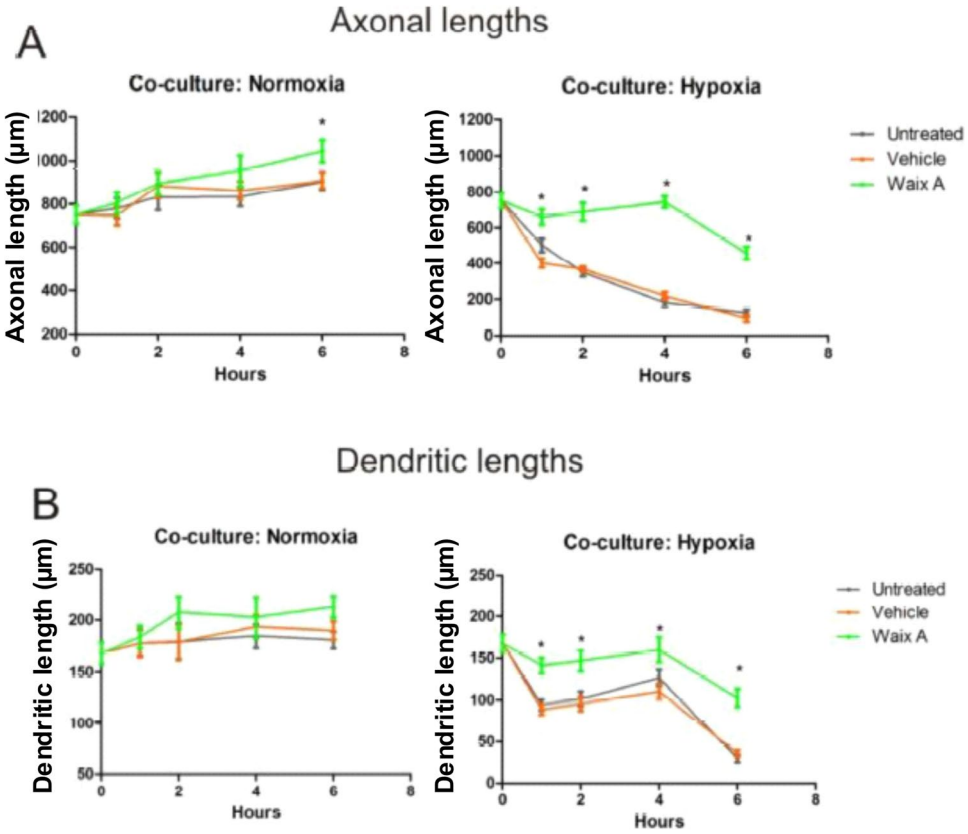

Fig. 7 Presence of astrocytes in culture exacerbates axonal retraction caused by hypoxia. **A** Axonal and **B** dendritic lengths of DIV4 neurons in neuron-astrocyte co-culture under normoxic and hypoxic conditions. All data are presented as mean ± SEM, $*p < 0.05$ (ANOVA with Bonferroni post hoc)

retraction with and without astrocytes present in culture. It should be noted that a limitation of the use of waixenicin A to examine TRPM7 activity is that off-target effects cannot be completely ruled out. However, the low dosage of waixenicin A (500 nM), the compatible results obtained when using the TRPM7 activator naltriben [31], and our published results pertaining to axonal outgrowth using genetic shRNA knockdown of TRPM7 had consistent results when compared with waixenicin A treatment [15]. Currently, the repertoire for selective and potent TRPM7 inhibitors remains limited [32], but future studies will aim to assess other pharmacological approaches to validate our present findings.

Short-Term Hypoxia Reduced TRPM7 Activity, Enhanced Axonal Outgrowth of Neuronal Cultures, and Activated MEK/ERK and PI3K/Akt Pathways

In cerebral ischemia/hypoxia, ionic imbalances such as calcium overload have been identified to underlie the molecular and cellular mechanism of the resulting cell death and brain injury. TRPM7 plays a critical role under hypoxic and ischemic conditions, as demonstrated with hypoxic–ischemic brain injury in mice [33] as well as in CA1 neurons in vivo [34]. Most recently TRPM7 was found to mediate neuronal cell death, mediating improved outcomes after hypoxia/ischemia with the administration of TRPM7 blocker waixenicin A [31]. These studies build the narrative of TRPM7's substantial role and potential as a therapeutic target for hypoxic–ischemic brain injury [35]. Here, we demonstrate the effects of hypoxia on neurite outgrowth on E16 hippocampal neuron cultures. Cultures were grown until DIV4 and then separated into three treatment groups (untreated, vehicle-treated, and waixenicin A-treated) and subjected to hypoxia (5% O2) as described previously [24]. In the pre-treatment paradigm, waixenicin A application took place immediately before the onset of hypoxia, and neurons were exposed to hypoxic conditions for 1, 2, 4, 6, 8, and 10 h. Pre-hypoxia control neurons were fixed on DIV4 immediately before the onset of hypoxia and were used as control. Neurons were fixed immediately after the hypoxia treatment, and axonal lengths and dendritic lengths were analyzed using ICC with confocal microscopy and SynD analysis routine (Fig. 10).

We identified that with the treatment of short-term hypoxia, axonal outgrowth was significantly enhanced compared to all treatment groups under normoxic conditions. While the literature is still mixed on this topic, our results were consistent with those in PC12 cells, which also demonstrated neurite outgrowth under hypoxia [23]. Further, ovine fetal brains showed increased IGF-1 receptor

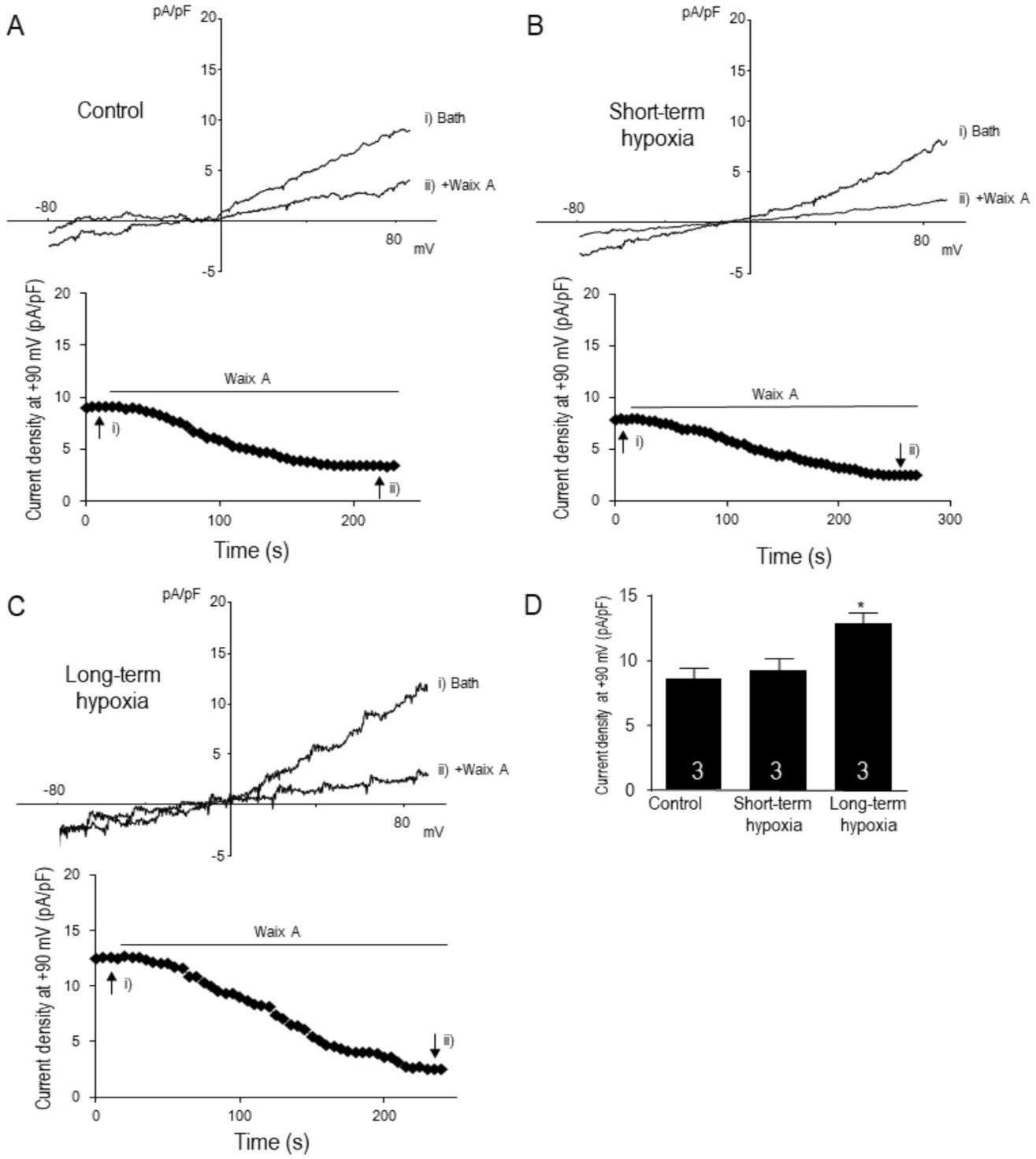

Fig. 8 When neurons were co-cultured with glia, neuronal TRPM7-like activity was enhanced with long-term hypoxia, but short-term hypoxia had no effect. Representative traces and time course of TRPM7-like current density before and after application of 500 nM waixenicin A and assessed at + 90 mV in **A** control (normoxic) neurons; **B** short-term hypoxic (~ 1 h in hypoxic chamber) neurons; and **C** long-term hypoxic (> 2.5 h in hypoxic chamber) neurons. Arrows on the time course indicate corresponding time points shown in traces. **D** Comparison of TRPM7-like current density (i.e., waixenicin A-sensitive inhibition component) in control (normoxic), short-term hypoxic, and long-term hypoxic neurons. Asterisk represents $p < 0.05$, comparison to control (one-way ANOVA with Bonferroni multiple comparison tests; $n = 3$/group). Bars represent SEM

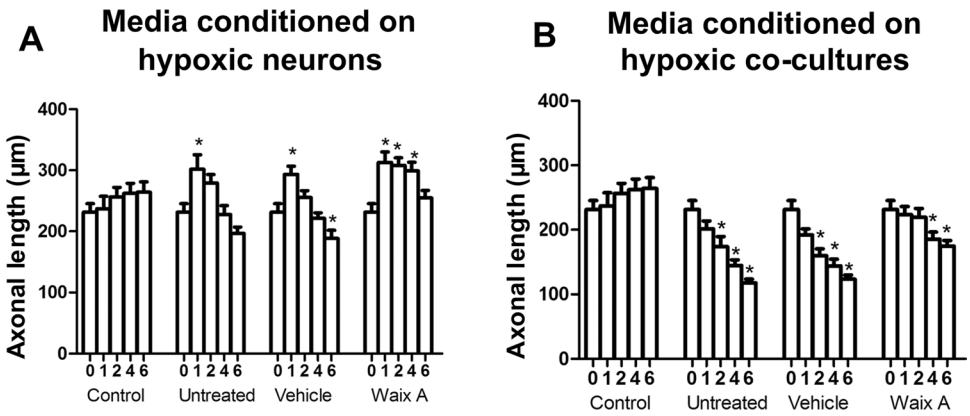

Fig. 9 Axonal lengths following a media switch. Axonal lengths of neurons that received conditioned media from **A** hypoxic neuronal cultures or **B** hypoxic neuron-astrocyte co-cultures. The numbers on *x*-axes represent the duration of hypoxia in hours. All data are presented as mean ± SEM, $*p < 0.05$ (ANOVA with Bonferroni post hoc)

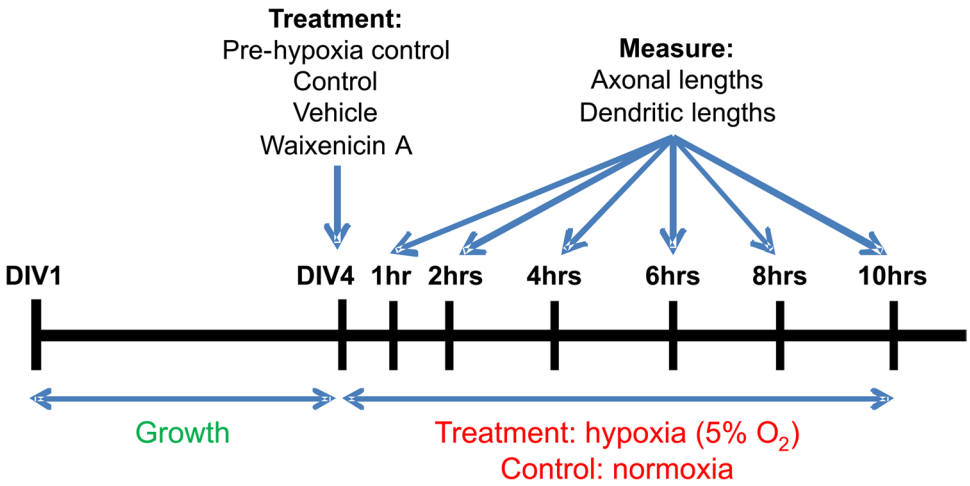

Fig. 10 The experimental timeline of hypoxia treatment

expression in the growth cone with hypoxia [36]. Our findings are also consistent with previously established literature identifying TRPM7 to be a negative regulator of axonal outgrowth [15].

Under hypoxic conditions, phospho-activation of MAPK/ERK kinase MEK1/2 and its downstream target ERK1/2 increased cell survival in mouse cultured cortical neurons through phospho-inactivation of pro-apoptotic Bcl2 family protein Bad [37]. In the neonatal brain, activation of ERK1/2 through the BDNF signaling pathway provided neuroprotection against hypoxic-ischemic injury [38]. Interestingly, a study on human umbilical vein endothelial cells (HUVECs) showed that both pharmacological inhibition of TRPM7 and downregulation of TRPM7 by siRNA enhanced cell growth and proliferation via activation of ERK signaling pathway [39]. Moreover, TRPM7 was also shown to regulate the proliferation of hepatic stellate cells via ERK and PI3K signaling pathways [40]. We have also recently demonstrated that ERK/MEK and PI3K/Akt signaling cascades are involved in TRPM7-mediated cytoskeletal regulation and migration of neuroblastoma cells [41]. These signaling cascades have also been shown to mediate neurite outgrowth.

Long-Term Hypoxia Potentiated TRPM7 Activity and Caused Retraction of Axons and Dendrites and Waixenicin A Attenuated This Retraction

Selectively pruning axons, dendrites, neurites, etc. occur in the natural development of the adult nervous system and underlies the basis of neuronal plasticity. Pruning can typically occur with either retraction or degeneration [42]. In comparison to axon growth, the mechanisms of axonal retraction remain relatively unclear. In literature, it has been suggested that axonal pathologies like retraction are the consequence of either an intrinsic program for retraction or inadequate support of the axonal cytoskeleton [42]. Previous literature has linked GTP-binding protein RhoA to axonal retraction [43, 44]. Where the activation of RhoA results in axonal retraction, where specifically a downstream effector of RhoA, Rho-associated coiled-coil forming kinase (ROCK) inhibition complete reverted retraction in hippocampal pyramidal neurons [45]. Recently in literature, the kinase of TRPM7 was found to phosphorylate RhoA and subsequently actin polymerization [46]. RhoA has been found to play a critical role in CNS injury, resulting in retraction and synaptic loss [47]. Inhibition of the

RhoA-ROCK signaling pathway was found to reverse the functional impairments caused by retraction/loss of dendritic spines in traumatic brain injury (TBI) [48]. LIM kinase (LIMK) is phosphorylated by ROCK, which phosphorylates cofilin, an actin-binding resulting in actin depolymerization. Suggesting a potential link between TRPM7 kinase activation, RhoA activation, and as a result retraction on axons and dendrites as found in our research.

Here, we find that long-term hypoxia potentiated the activity of TRPM7, and with the use of the specific blocker, waixenicin A was able to reverse the retraction that results from hypoxia. This remains consistent with other studies that found chronic hypoxia to cause dendritic retraction in the phrenic nucleus [49].

The Presence of Astrocytes Enhanced the Axonal Retraction in Hypoxic Conditions, Long-Term Hypoxia Potentiated TRPM7 Activity

Complex communication between neurons and other cell types like astrocytes is required to coordinate events within the cellular network. Important mediators of cell-to-cell contact are called cell adhesion molecules (CAMs). There are two key elements in the communication between neurons and astrocytes that have been identified within the literature: αvβ3 integrin and the receptor Thy-1 [50]. Thy-1 is a 25–37 kDa glycosylphosphatidylinositol (GPI)-anchored protein expressed in various cell types and is critical to cell death, signaling motility, and adhesion. Thy-1 then can bind to integrins $\alpha_v\beta_3$, which is ubiquitously expressed [51]. The binding of $\alpha_v\beta_3$ and Thy-1 results in integrin clustering and activation of the RhoA-ROCK signaling pathway. Recently, it was identified that the integrin-engaged Thy-1 complex causes neurite retraction via the RhoA/ROCK pathway, as Thy-1 clustering triggers actin cytoskeleton remodeling [52]. As it has previously been identified that hypoxia induces an increase in intracellular magnesium via TRPM7, and since the kinase activity of TRPM7 requires magnesium, its activity may be affected by phosphorylated substrates under hypoxic conditions [53]. As mentioned previously, RhoA has been discovered to be a substrate of the TRPM7 kinase, aligning with our results hypothesizing a possible pathway of increased activation of the TRPM7 kinase resulting in increased phosphorylation of RhoA and subsequently actin polymerization and neurite retraction.

In conclusion, our study investigates the effects of hypoxia on TRMP7 mediated neurite outgrowth and opens the field for investigating potential mechanisms and signaling pathways of neurite outgrowth and retraction. These findings provide elaborated information on the potential therapeutic potential of TRPM7 as a target for disrupted neuronal growth, retraction, and regeneration in hypoxia.

Author Contribution All authors contributed to the study. Material preparation, data collection, and analysis were performed by ET, MD, and RW. All authors listed made direct and substantial contribution to this manuscript. All authors read and approved the final manuscript. All authors discussed the results, analyzed data, and commented on the manuscript; HSS and ZPF designed and developed the study.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval Primary cell culture protocol has been approved by the University of Toronto Animal Care Committee.

Consent to Participate All authors agree in participation of the research project and publication of the research findings.

Consent for Publication All authors have agreed to publish the research findings.

Competing Interests The authors declare no competing interests.

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