Analysis of TRP channel functions in sensory neurons and microglia

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(Summary)

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Overall introduction

Sensing is a fundamental ability of all the organisms. Regardless of the character of the signals we sense, they bring us beneficial information to make decisions, such as 'Go' or 'No Go'. Similarly, sensing environmental changes is an essential function at the cellular level. Transient receptor potential (TRP) channels are involved in a wide variety of sensing functions in the human body, including pain and temperature sensations. The TRP channel superfamily is conserved in yeast, invertebrates and vertebrates and composed of 28 members which are divided into 6 subfamilies in mammals, based on their protein sequence homology: canonical or classic (C), vanilloid (V), melastatin (M), polycystin or polycystic kidney disease (P), mucolipin (ML), ankyrin (A). The mechanisms activating TRP channels are highly diversified: activation by temperature, by chemical molecules or activation downstream of signal transduction pathways. Additionally, it is well known that TRP channels are polymodal receptors. The TRP channel activators cause synergistic effects on TRP channel activity when different types of activators exist at the same time. Thus, TRP channels attract us to study about their physiological and pathological significance involved in our lives. In this doctoral thesis study, I focused on TRP channel functions involved in the mechanism of pain sensation induced by propofol, a general anesthetic drug (Chapter I) and the mechanism of temperature-dependent microglial motility (Chapter II).

Chapter I.

Propofol-induced pain sensation involves multiple mechanisms in sensory neurons

Introduction

Propofol, a commonly used intravenous anesthetic agent, is known to sometimes cause pain sensation upon injection in humans. However, the molecular mechanisms underlying this effect are not fully understood. Although propofol was reported to activate human TRP ankyrin 1 (TRPA1), its action on human TRP vanilloid 1 (TRPV1), another nociceptive receptor, is unknown. Furthermore, whether propofol activates TRPV1 in rodents is controversial. In this study, I tried to clarify the molecular mechanisms of propofol-induced pain sensation by focusing on TRPA1, TRPV1 and γ-amino butyric acid ($GABA_A$) channels. The results were published (Nishimoto *et al.*, 2015).

Materials and Methods

All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences and carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals (NIH publication no. 85-23. Revised 1985).

Animals

C57BL/6NCr (Wt) mice (5- to 8-weeks-old, SLC) were used as a control. *Trpv1*/*Trpa1*

double-knockout (V1A1DKO) mice were obtained from a mating between *Trpv1*-knockout (V1KO) and *Trpa1*-knockout (A1KO) mice (both were generously provided by Dr. David Julius, UCSF, San Francisco, CA, USA) (Caterina *et al.*, 2000; Bautista *et al.*, 2006), which were backcrossed on a C57BL/6NCr background. Mice were housed in a controlled environment (12 hrs light/12 hrs dark cycle; room temperature, 22 to 24°C; 50 to 60% relative humidity) with free access to food and water. The genotyping of V1A1DKO mice used for the experiments was performed by PCR.

Isolation of dorsal root ganglion (DRG) cells

Mouse DRG (at thoracic and lumber levels) from each genotype were rapidly dissected and dissociated by incubation at 37°C for 20 min in a solution of culture medium that contained Earle's balanced salts solution (Sigma-Aldrich), FBS (10%, BioWest or Gibco), penicillin-streptomycin (50 mg/mL and 50 units/mL respectively, Gibco), GlutaMAX (2 mM, Gibco), and vitamin solution (1%, Sigma-Aldrich), with 0.25% collagenase type XI (Sigma-Aldrich). Cells were gently triturated using fire-polished Pasteur pipettes and centrifuged in culture medium to separate cells from debris. Cells were resuspended and plated onto 12-mm cover slips coated with poly-D-lysine (Sigma-Aldrich). The patch-clamp recordings were performed 12 to 24 hrs after the incubation of isolated DRG cells as described below. Ca^{2+}-imaging experiments were performed 12 to 20 hrs after the incubation of isolated DRG cells as described below.

Electrophysiology

HEK293T cells and isolated mouse DRG cells were used for patch-clamp recordings.

HEK293T cells were maintained in D-MEM (Wako) supplemented with 10% FBS (BioWest), penicillin-streptomycin (50 mg/mL and 50 units/mL, respectively, Gibco), and GlutaMAX (2 mM, Gibco) and seeded at a density of 5×10^5 cells per 35-mm dish 24 hrs before transfection. For patch-clamp recordings of HEK293T cells, either 1 μg human TRPA1 (hTRPA1), human TRPV1 (hTRPV1), mouse TRPV1 (mTRPV1), or mouse TRPA1 (mTRPA1) channel expression vector and 0.1 μg pGreen-Lantern 1 vector were transfected into HEK293T cells using Lipofectamine and Plus reagents (Invitrogen). Patch-clamp recordings of HEK293T cells were performed 18 to 36 hrs after the transfection. Mouse DRG cells were prepared as described above. The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose at pH 7.4 adjusted with NaOH. The intracellular solution for the experiments with HEK293T cells contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES at pH 7.4 adjusted with KOH. For the recordings of mouse DRG cells, the intracellular solution contained 67 mM KCl, 65 mM K-gluconate, 1.0055 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM Mg-ATP, 1 mM 2Na-GTP, 5 mM EGTA, and 10 mM HEPES at pH 7.3 adjusted with KOH. The free Ca^{2+} concentration was 20 nM (calculated with CaBuf; www.kuleuven.be/fysio/trp/cabuf). A Ca^{2+}-free bath solution used in the Ca^{2+}-free experiments was made by removing 2 mM $CaCl_2$ and adding 5 mM EGTA to the standard bath solution. Data for analysis were sampled at 10 kHz and filtered at 5 kHz for whole-cell recordings and 2 kHz for single-channel recordings (Axopatch 200B amplifier with pCLAMP software, Molecular Devices). In the experiments with mouse DRG cells at a current-clamp mode, the cells in which the resting potential was under -40 mV were selected. All of the patch-clamp experiments were performed at room temperature. The coverslips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical

stimulation was applied by running a bath solution containing various chemical reagents.

Ca^{2+}-imaging

Mouse DRG cells on coverslips were incubated at 37°C for 30 min in culture medium containing 5 μM Fura-2-acetoxymethyl ester (Molecular Probes). The cover slips were washed with a standard bath solution identical to the extracellular solution in the patch-clamp recordings and a Ca^{2+}-free bath solution identical to the extracellular solution used in the patch-clamp experiments. Fura-2 fluorescence was measured in a standard bath solution. Fura-2 was excited with 340- and 380-nm wavelength lights and the emission was monitored at 510 nm with a CCD camera, CoolSnap ES (Roper Scientific/Photometrics) at room temperature. Chemical stimulations were applied as described above for the patch-clamp recordings. Data were acquired using IPlab software (Scananalytics) and analyzed with ImageJ software (National Institutes of Health) and Excel software (Microsoft). Ionomycin (5 μM, Sigma-Aldrich) was applied to confirm cell viability and values were normalized to those evoked by ionomycin for each experiment. Cells in which an increase in normalized intensity during propofol application was over 0.2 were considered activated.

Chemicals

Chemicals used in this study were purchased as described below. Propofol (2,6-diisopropylphenol), HC-030031, capsaicin, ionomycin, nifedipine, γ-amino butyric acid (GABA), and (+)-bicuculline were from Sigma-Aldrich. Allyl isothiocyanate (AITC) was from Kanto Chemical. Picrotoxin, verapamil hydrochloride, flunarizine dihydrochloride and NNC 55-0396 dihydrochloride were from Tocris. Propofol, picrotoxin, flunarizine, nifedipine,

and (+)-bicuculline were dissolved in DMSO as stock solutions. Capsaicin and AITC were dissolved in ethanol and methanol, respectively. The others were dissolved in water. All of the dissolved chemicals were diluted (from 1:10000 to 1:1000) into the solution for the patch-clamp and Ca^{2+}-imaging experiments. The concentration of DMSO did not exceed 0.15%.

Statistical analysis

Data are presented as means ± standard error of mean (SEM). The abbreviation n indicates the number of data points. The Mann-Whitney *U* test, unpaired *t*-test, Chi-square test, and non-parametric multiple comparison were applied for statistical analyses. P values less than 0.05 were considered significant. Data from the propofol-evoked hTRPA1 current recordings were fitted with a Hill's equation to generate a dose-response curve and EC_{50} values were calculated. Data related to the inhibitory effect of HC-030031 on propofol-evoked humanTRPA1 currents were fitted by a logistic curve to calculate the IC_{50} value. All statistical analyses were performed using Origin software (OriginLab).

Results

Patch-clamp studies of propofol-evoked TRP channel currents

First, I utilized a patch-clamp method to examine propofol actions in HEK293T cells expressing either hTRPA1, mTRPA1, hTRPV1 or mTRPV1. 100 μM of propofol activated both hTRPA1 and mTRPA1 with an outwardly rectifying current-voltage relationship although an increase in

currents upon propofol washout was observed in the case of mTRPA1, probably due to the bimodal effects of the compound on mTRPA1. Once large current responses were observed upon propofol application, the following AITC responses were small. Propofol-evoked current activation was observed in relation to mTRPV1, but not hTRPV1, although both hTRPV1 and mTRPV1 responded similarly to capsaicin (Cap, 1 μM) with clear outward rectification in the same cells, indicating that propofol actions on TRPV1 differ depending on species.

Next, I tried to determine the dose-dependency for the propofol effects on the four TRP channels. When I analyzed propofol-evoked currents during the propofol application, I found that propofol was the most effective at hTRPA1, in which propofol effects were almost saturated at 100 μM. The effects of propofol on mTRPA1 exhibited a bell-shape curve, possibly due to its bimodal action. High concentrations of propofol caused small but significant mTRPV1 activation at -60 mV although the current activation looked negligible. When the curve of the dose-dependent activation of hTRPA1 by propofol was fitted with a Hill equation, Hill co-efficient and EC_{50} value were 3.3 and 65.4 μM, respectively, representing attainable concentrations in the clinical setting (Doenicke *et al.*, 1996). 100 μM propofol did not cause measurable hTRPA1 activation at -60 mV in the absence of extracellular Ca^{2+}. Propofol-evoked currents were inhibited reversibly by HC-030031, a specific TRPA1 antagonist, with an IC_{50} value of 1.2 μM.

In order to examine whether hTRPA1 is directly activated by propofol in a membrane-delimited manner, I performed single-channel recordings in an inside-out mode of a membrane excised from a HEK293T cell expressing hTRPA1. Clear single-channel openings at a membrane potential of +60 mV were observed upon application of propofol (30 μM), and robust hTRPA1

channel activation by 30 μM AITC, confirming the hTRPA1 activation by propofol, which indicate that propofol can activate hTRPA1 directly and that the intracellular component is not necessary for the mechanism of propofol-induced hTRPA1 activation.

The effects of propofol on mouse DRG cells

Thus far, propofol was shown to directly activate hTRPA1 at a concentration attainable in a clinical setting. To further confirm the ability of propofol to activate TRPA1, I performed Ca^{2+}-imaging experiments using mouse DRG cells. A previous report showed that propofol-induced $[Ca^{2+}]_i$ increases were not observed in TRPA1-deficient DRG cells while another report showed that propofol-induced $[Ca^{2+}]_i$ increases were still observed in DRG cells from V1A1DKO mice . Some DRG cells from Wt mice responded to propofol (50 μM), and a greater number of cells responded to AITC (100 μM) and/or Cap (1 μM). Propofol induced $[Ca^{2+}]_i$ increases through Ca^{2+} influx from outside of the cells. Next, in order to confirm the involvement of TRPV1 and/or TRPA1 in the propofol-induced $[Ca^{2+}]_i$ increases, I compared the propofol-induced $[Ca^{2+}]_i$ increases in DRG cells from Wt, A1KO, V1KO and V1A1DKO mice. The propofol-induced $[Ca^{2+}]_i$ increases were observed in A1KO, V1KO and V1A1DKO DRG cells although the percentage of the propofol-responsive DRG cells was significantly smaller in V1A1DKO compared with Wt, A1KO and V1KO DRG cells. This result suggests that propofol actions on mouse DRG cells are almost similar even if either TRPA1 or TRPV1 is genetically abolished, and that both TRPA1 and TRPV1 might be involved in propofol-induced $[Ca^{2+}]_i$ increases.

Involvement of $GABA_A$ receptors and voltage-gated Ca^{2+} channels in propofol-induced

$[Ca^{2+}]_i$ increases in V1A1DKO DRG cells

Because propofol is known to act on $GABA_A$ receptors (Orser *et al.*, 1994), $GABA_A$ receptor activation by propofol in DRG cells could cause depolarization. It is well known that intracellular chloride concentrations in DRG cells are quite high due to the lack of potassium chloride cotransporter 2 (KCC2) expression (Mao *et al.*, 2012). Therefore, I hypothesized that depolarization involving $GABA_A$ receptor activation would activate voltage-gated Ca^{2+} channels to cause $[Ca^{2+}]_i$ increases upon propofol application, although such membrane depolarization should also activate voltage-gated Na^+ channels. In order to confirm the hypothesis, I performed Ca^{2+}-imaging experiments using DRG cells from V1A1DKO mice, the use of which excludes the involvement of TRPA1 or TRPV1 in propofol-induced $[Ca^{2+}]_i$ increases. Propofol-induced $[Ca^{2+}]_i$ increases were drastically and reversibly inhibited by picrotoxin (Pic, 100 μM), a $GABA_A$ receptor antagonist. Another $GABA_A$ receptor antagonist, (+)-bicuculline (Bic, 30 μM), also inhibited propofol-induced $[Ca^{2+}]_i$ increases almost completely, indicating that a majority of the TRPV1/TRPA1-independent component of the propofol-induced $[Ca^{2+}]_i$ increases is caused by $GABA_A$ receptor activation. Among the voltage-gated Ca^{2+} channels that could be activated by depolarization downstream of $GABA_A$ receptor activation, I examined the effects of L-type voltage-gated Ca^{2+} channel inhibitors, verapamil (Ver) and nifedipine (Nif), and T-type voltage-gated Ca^{2+} channels inhibitors, flunarizine (Flu) and NNC 55-0396 (NNC), on propofol-induced $[Ca^{2+}]_i$ increases. All of them inhibited propofol-induced $[Ca^{2+}]_i$ increases, suggesting that both L-type and T-type voltage-gated Ca^{2+} channels are activated by depolarization upon $GABA_A$ receptor activation.

Propofol-induced depolarization of mouse DRG cells through $GABA_A$ receptor activation

Given that propofol exhibited an ability to cause intracellular $[Ca^{2+}]_i$ increases in DRG cells though $GABA_A$ receptor activation, I performed patch-clamp recordings of GABA-responsive V1A1DKO DRG cells to confirm whether $GABA_A$ receptor activation by propofol causes depolarization of DRG cells. Not only GABA but also propofol depolarized isolated V1A1DKO DRG cells followed by action potential generation, whereas such propofol-induced action potential generation observed in V1A1DKO DRG cells was inhibited by picrotoxin. These results obtained in my preparations indicate that $GABA_A$ receptor activation by propofol causes action potential generation in mouse DRG cells, suggesting that TRPA1 and TRPV1 are not the sole targets for propofol actions.

Discussion

In this study, I found that propofol activated human and mouse TRPA1. In contrast, I did not observe propofol-evoked human TRPV1 activation, whereas the ability of propofol to activate mouse TRPV1 was very small. I also found that propofol caused increases in intracellular Ca^{2+} concentrations in a considerable portion of DRG cells from mice lacking both TRPV1 and TRPA1, indicating the existence of TRPV1- and TRPA1-independent mechanisms for propofol action. In addition, propofol produced action potentials in a manner dependent on a type A $GABA_A$ receptor. Finally, I found that both T-type and L-type voltage-gated Ca^{2+} channels were activated downstream of $GABA_A$ receptor activation by propofol. Thus, propofol may cause pain sensation through multiple mechanisms involving not only TRPV1 and TRPA1 but also voltage-gated channels downstream of $GABA_A$ receptor activation. These findings might provide effective approaches for the prevention of propofol-induced pain sensation and the

development of treatments.

Chapter II.

Temperature-dependent microglia movement and the involvement of thermosensitive TRP channels

Introduction

Microglia are resident immune cells in the brain that play important roles in its maintenance. Under physiological conditions, they survey the surrounded area where neurons and other glial cells exist with highly motile processes. They also transform into the activated phenotype in response to the environmental changes under pathological conditions such as brain damage, injury or inflammations. Therapeutic hypothermia is an effective treatment for neural protection in the clinical field and also suppresses microglial functions such as cytokine release. However, there are few studies focusing on the molecular basis involved in the changes of microglial function in therapeutic hypothermia. Some of thermosensitive TRP channels are reported to function in microglia although no one focused on their thermal sensitivity in microglial function. Therefore, I tried to evaluate the involvement of thermosensitive TRP channels in microglial function, especially microglia movement, using temperature-controlled time-lapse imaging system, patch-clamp recordings and molecular techniques.

Materials and methods

All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences and carried out in

accordance with the National Institutes of Health Guide for the care and use of laboratory animals (NIH publication no. 85-23. Revised 1985).

Animals

C57BL/6NCr (Wt) mice (SLC, Hamamatsu, Japan) were used. They were housed in a controlled environment (12 hrs light/12 hrs dark cycle; room temperature, 22 to 24°C; 50 to 60% relative humidity) with free access to food and water.

Cell culture

Primary mouse microglia were obtained according to the method descried previously (Koizumi *et al.*, 2007; Doering, 2010) with modification. In brief, a glia mixed culture was prepared from postnatal (P0-P1) pups. Dissected cortical hemispheres were triturated using a fire-polished Pasteur pipette and filtered through 100 μm pore nylon mesh. Cells were resuspended in D-MEM (D6046, Sigma-Aldrich) containing 10% (v/v) heat-inactivated bovine serum (Sigma-Aldrich), penicillin-streptomycin (10 units/mL and 10 mg/mL, respectively, Gibco), bovine insulin (5 μg/mL, Sigma-Aldrich) and glucose solution (2 mg/mL, Otsuka Pharmaceutical Co., Ltd.) (microglia medium) and cultured in 75-cm^2 tissue flasks at 4 x 10^6 to 8 x 10^6 cells/flask in fresh microglia medium (glia mixed culture). After cultivation for 10 to 20 days at 37°C in a humidified CO_2 incubator, microglia were isolated by shaking the flasks of glia mixed cultures at 37°C at 80 rpm for 60 to 120 min on an orbital shaker. Cells were used within 6 days after isolation for each experiment and the medium was changed every 3 days for further incubation.

Time-lapse imaging and quantitative analysis of microglia movement

Primary mouse microglia on glass bottom dishes were incubated for 2 to 6 days before use. Time-lapse imagings were performed with Keyence BZ-9000 (Keyence) fitted with a temperature-controlled stage incubator (Tokai Hit) under a circulating mixture of gases (20% O_2/5% CO_2/75% N_2). The image acquisition was performed using BZ-9000 software with a Z-stack function. For assays of microglia movement, the stacking images were processed and analyzed by a newly developed cell-tracking program based on template matching, a digital processing technique used for pattern recognition and object searching. The *xy* coordinates elicited from the cell-tracking program were further analyzed using ImageJ software (National Institutes of Health), Chemotaxis and migration tool plug-in and Excel software (Microsoft) to calculate the migrating distances of an individual cell. Data defining the migrating distances of microglia were collected from at least 2 individual glass bottom dishes in 2 different preparations.

Electrophysiology

Primary mouse microglia on cover glasses were incubated in culture medium at 37^{o}C. The cover glasses were washed with a standard bath solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose at pH 7.4 adjusted with NaOH. After achievement of a whole cell configuration, the solution was replaced with K^+-free bath solution containing 145 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose at pH 7.4 adjusted with NaOH. The intracellular solution for whole-cell recording contained 20 mM NaCl, 120 mM Na gluconate, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES at pH 7.3 adjusted with NaOH. For the experiments in which the concentration of intracellular

free calcium ($[Ca^{2+}]_i$) was fixed to 1 μM, 4.53 mM $CaCl_2$ was added (calculated by Maxchelator, standard version, http://maxchelator.stanford.edu /downloads.htm) and then the pH was adjusted. Data for analyses were sampled at 10 kHz and filtered at 5 kHz for whole-cell recordings (Axon 200B amplifier with pCLAMP software, Molecular Devices). Thermal stimulation was applied by increasing the bath temperature with a preheated solution through an inline heater (SH-27B, Warner Instruments). The bath temperature in a chamber during recordings was monitored with a thermocouple (TA-29, Warner Instruments) and sampled with an analog-to-digital converter with pCLAMP software (Molecular Devices). Membrane potential was clamped at -70 mV for primary microglia. Ramp pulses from -100 to +100 mV for 500 msec were applied every 5 sec. All the experiments were performed at room temperature except for the experiments of thermal stimulation.

Immunostaining

Primary mouse microglia were used within 6 days of culture on a glass bottom dish. Cells were fixed by using ice-cold 4% paraformaldehyde (PFA) for 10 min. Non-specific immunoreactive sites were blocked with 1% bovine serum albumin (Sigma-Aldrich) in PBST (PBST-BSA) for 1 hr. Cells were incubated overnight at 4^{o}C with primary antibodies against integrin β2 (CD11b, AbD Serotec, 1:50) and TRPM4 (a gift from Dr. Naruse, Okayama university). Cells were incubated for 1 hr with goat anti-rabbit Alexa Fluor 488 and goat anti-rat Alexa Fluor 568 (Thermo Fisher Scientific, 1:2000). Cells were incubated with 4', 6-diamidino-2-phenylindole (DAPI, Dojindo, 1:1000) in PBST for 10 to 15 min to stain the nucleus. All the procedures were conducted at room temperature except for the reaction of primary antibodies.

RT-PCR and quantitative RT-PCR

Total RNA was purified from primary mouse microglia using an RNeasy Plus Mini kit (RT-PCR) or an RNeasy Micro Kit (qRT-PCR) (Qiagen) with DNase I treatment for elimination of genomic DNA according to the manufacturer's protocols. cDNA was synthesized from total RNAs (up to 1 μg) using reverse transcription with a Superscript III first-strand synthesis system for reverse transcription (RT)-PCR (Invitrogen). PCR was performed using rTaq polymerase (TaKaRa) in the iCycler (Bio-Rad) with primer sets (5'-TGAAGGGTGGAGCCAAAAGG-3' and 5'-GGAAGAGTGGGAGTTGCTGTTG-3' for *Gapdh*, 5'-CGGCTTCAGAGATGACCAG-3' and 5'-GCTTCATTCATCATGTCCTTG-3' for *Cd11b*, 5'-TGGATGCTCTGCTGAATGAC-3' and 5'-GACTCTAGGCGAGCCATCAC-3' for *Trpm4*).

Statistical analysis

Data are presented as means ± standard error of mean (SEM). The abbreviation n indicates the number of data points. The one-way analysis of variance (ANOVA) with Tukey-Kramer test and non-parametric multiple comparison with Shirley-Williams test were applied for statistical analyses. P values less than 0.05 were considered significant. All statistical analyses were performed using OriginPro software (OriginLab) or Excel add-in software, Statcel 4 (OMS, Ltd.).

Results

To address whether microglia movement was affected by temperature, microglia movements were monitored at a single-cell level with a time-lapse phase-contrast microscopy. I found that microglia movement is temperature-dependent. In addition, I found suggestions that TRPM4 activation was involved in the temperature-dependent microglia movement. First, I observed TRPM4 expression in mouse microglia not only at the mRNA level by RT-PCR but also at the protein level by immunocytochemistry. Next, I performed patch-clamp experiments to examine whether TRPM4 can function in microglia. Heat-evoked currents were observed in isolated microglia and 9-phenanthrol, a TRPM4 inhibitor, inhibited such currents in a dose-dependent manner. Finally, I observed that the temperature-dependency of microglia movement was diminished by 9-phenanthrol treatment. These data indicated that TRPM4 is functionally expressed in microglia and could be involved in temperature-dependent microglia movement.

Discussion

In the present study, I found that microglia movement is clearly temperature-dependent and that TRPM4 channels among thermosensitive TRP channels are possibly involved in the temperature-dependent microglia movement.

In the brain, microglia as resident immune cells exhibit a robust motile feature that characterizes them as cells distinct from other glial cells such as astrocytes and oligodendrocytes (Hanisch *et al.*, 2007). In response to the brain damage or inflammation, surveillant microglia transform into the activated phenotype to migrate toward the damaged area and release various molecules including cytokines. Although there is a work showing that microglial motility was different

between 33°C and 37°C conditions with regard to speed of microglia process movement, the difference was reported to be caused by the temperature-dependence of neuronal activity. Moreover, the temperature-dependency was lost in the presence of tetrodotoxin (TTX) (Wake *et al.*, 2009). In the ischemic situation, hypothermia protects neuronal functions by suppressing the immune responses stimulated by ischemic damage (Yenari *et al.*, 2012). Microglia migrate to the damaged area and release pro-inflammatory cytokines and reactive oxygen species (ROS) that can activate immune cells invading the brain. My analysis of temperature-dependent microglia movement suggests that therapeutic hypothermia can suppress the microglia movement as well as cytokine production. Thus, my results in this study will help to understand the basis of cell motility for the establishment of better applications in therapeutic hypothermia while avoiding the side effects.

Given that microglia movement was temperature-dependent, I examined the mRNA expression levels of several thermosensitive TRP channels whose temperature thresholds are within the body temperature range, and found the expression of TRPM4 mRNA as a candidate. I examined the involvement of TRPM4 in microglia movement using a pharmacological approach with a patch-clamp method and time-lapse imaging technique. I observed the expression of TRPM4 in isolated microglia at the protein level. In my analysis of TRPM4 function, 9-phenathrol inhibited both heat-evoked currents in isolated microglia and temperature-dependent microglia movement. These results suggest the involvement of TRPM4 in the temperature-dependent microglia movement.

In conclusion, I identified TRPM4 as an important molecule involved in the temperature-dependent movement of mouse microglia. Although further studies are necessary to confirm this concept, modulation of TRPM4 function in microglia could lead to the

development of therapeutic ways to treat various diseases that involve TRPM4.

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