Physiological significance of TRPV4 channels in mouse Schwann cells

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Introduction

Schwann cells (SCs) are the primary glial cells of the peripheral nervous system. Myelinating SCs wrap around large-diameter axons and form the myelin sheath. While many transcription factors and signaling molecules are involved in SC myelination, calcium signaling has been found to be an important mediator of this process. Transient receptor potential vanilloid 4 (TRPV4), a member of the TRP channel family, is a non-selective calcium-permeable cation channel. TRPV4 is expressed and activated throughout the body by various stimuli including mechanical stimulation, moderate heat, osmolarity and some endogenous or exogenous chemicals. According to recent reports, this channel was found to be widely expressed and functional in various glial cells, including astrocytes, microglia, oligodendrocytes and satellite glial cells. However, whether TRPV4 is expressed and functional in SCs or not remains unclear.

Materials and methods

Mice 15-week-old C57BL/6NCr male and female mice were used. Mice were kept under a 12-hour light-dark cycle, at 24°C with unlimited food and water. TRPV4-deficient (TRPV4KO) mice were maintained on a C57BL6/NCr background (54).

Chemicals GSK1016790A (GSK), capsaicin, pregnenolone sulfate (PS), lysophosphatidylcholine (LPC) and ionomycin calcium salt were purchased from Sigma-Aldrich; Allyl isothiocyanate (AITC) was purchased from KANTO; Camphor was purchased from Wake; Fura-2 was purchased from Invitrogen.

Isolation and purification of mouse Schwann cells (SCs) Mouse SCs from postnatal day 1-3 (P1-3) mice were established according to a previously reported method (55). After the SC cultures had reached approximately 80% confluence, contaminating fibroblasts were removed by a complement reaction using Thy 1.1 antibodies (58).

RT-PCR RT-PCR was performed as previously reported (59). Total RNA was isolated from purified primary SCs using Sepasol-RNA I super G (09379-84, Nacalai Tesque). Reverse transcription was performed using Super Script III reverse transcriptase (18080-085, Invitrogen) according to the manufacturer's instructions. RNA concentration and quality were assessed using a Nanodrop (Isogen Life Science, Belgium). Primers designed using pick primers from the National Center for Biotechnology Information.

Immunostaining Cells or tissues were incubated with blocking buffer (PBS containing 0.25% Triton X-100 and 1% bovine serum albumin) for 30 min at room temperature (RT), then incubated overnight at 4°C with primary antibody (diluted in blocking buffer). After 3 washes with blocking buffer, secondary antibody (1:1000, diluted in blocking buffer) was applied for 1 h at RT, followed by 3 washes with PBS. Images were obtained with a fluorescence microscope (BZ9000; Keyence, Osaka, Japan) or laser-scanning confocal microscope (LSM-510; Carl Zeiss). The following primary antibodies were used: anti-S100 antibody (1:200; ab868, Abcam), anti-rabbit Myelin Protein Zero (1:200; ab31851, abcam), anti-rat myelin basic protein (1:200; MAB386, Merk Millipore), anti-mouse MAG (1:300; sc-166848, Santa Cruz Biotechnology), anti-chicken GFAP (1:400; ab4674, Abcam), and anti-rabbit TRPV4 (1:500; CB-ACC-034, Alomone). The following secondary antibodies were used: goat anti-rat IgG Alexa Fluor® 488 (a11006, Invitrogen), goat anti-chicken IgY (H+L) Alexa Fluor® 647 (ab150171, Abcam), goat anti-chicken IgY (H+L) Alexa Fluor® 488 (A11039, Invitrogen), donkey anti-rabbit IgG Alexa Fluor® 488 (A21206, Invitrogen), and goat anti-mouse IgG (H+L) Alexa Fluor® 546 (A11030, Invitrogen).

Western blotting Total protein was extracted from purified SCs or sciatic nerves. The following primary antibodies were used in the western blots: anti-rabbit Myelin Protein Zero (ab31851, abcam), anti-rat myelin basic protein (MAB386, Merk Millipore), anti-mouse MAG (sc-166848, Santa Cruz Biotechnology), anti-rabbit TRPV4 (CB-ACC-034, Alomone), anti-rabbit EGR2 (ab108399, abcam), anti-rabbit c-Jun mAb (9165, Cell Signaling Technology), and anti-mouse vinculin antibody (VIN-11-5, Sigma). The following secondary antibodies were used for western blotting: HRP-conjugated GAPDH rabbit mAb (8884, Cell Signaling Technology), HRP-linked anti-rabbit IgG (7074, Cell Signaling Technology), HRP-linked anti-mouse IgG (7076, Cell Signaling Technology), and HRP-linked anti-rat IgG (112-035-062, Jackson).

Calcium imaging Purified SCs were cultured on poly-L-lysine/laminin-coated glass coverslips. After loading with 5 μM Fura-2-acetoxymethyl ester (Fura-2) for 1 h, SCs were mounted in an open chamber and superfused with bath solution. The standard bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM glucose, and pH 7.4 adjusted with NaOH. For Ca^{2+}-free bath solution, 5 mM EGTA was added instead of 2 mM $CaCl_2$. All chemicals were dissolved in the standard bath solution. For Ca^{2+}-free experiments, chemicals were dissolved in Ca^{2+}-free bath solution. All experiments were performed at room temperature unless otherwise

stated. For thermal stimulation experiments, SCs were placed at 33°C in a 5% CO_2 humidified incubator 24 h before Fura-2 loading. Thermal stimulation was performed by increasing the bath temperature using pre-warmed standard bath solution (~ 37°C). The temperature was monitored using a thermocouple (TC-344; Warner Instruments, Hamden, CT, USA) placed into the bath. Cytosolic free Ca^{2+} concentrations were measured by dual-wavelength Fura-2 microfluorometry with excitation at 340/380 nm and emission at 510 nm. The ratio image was calculated and acquired using the IP-Lab image processing system (Scanalytics, Milwaukee, WI, USA).

Patch-clamp recording Purified SCs were cultured on glass coverslips. Patch pipettes were made from borosilicate glass (type 8250, Garner Glass Company, United States) using a five-step protocol and a P-97 micropipette puller (Sutter Instrument, United States), with a tip resistance of 4 to 6 MΩ. Currents were recorded using an Axopatch 200B amplifier (Molecular Devices, United States) and filtered at 5 kHz with a low-pass filter. Currents were digitized with Digidata 1440A (Axon Instruments, United States). Data acquisition was achieved with pCLAMP 10 software (Axon Instruments, United States). The standard bath solution was the same as described for the calcium imaging. The cesium chloride pipette solution contained 140 mM CsCl, 5 mM EGTA, 10 mM HEPES, pH 7.40 with CsOH. The holding potential was -60 mV, and the ramp-pulse was from -100 to +100 mV for a 300 ms duration. All data and graphs were statistically analyzed using Origin Pro8 (OriginLab, United States).

Sciatic nerve cut injury Sciatic nerve surgeries were performed as reported previously (27). Briefly, mice were anesthetized by isoflurane, the right sciatic nerve was exposed and cut at the sciatic notch (distal stump), then the skin was closed. The distal stumps which were at a 1 mm distance from the cut site were collected for analysis at various time points.

Sciatic nerve cut injury in vitro This experiment was performed by modification of a previously reported method (17). Sciatic nerves from 15-week-old WT and TRPV4KO mice were isolated, cut into 5 mm segments, and seeded onto a 35 mm plate and cultured in DMEM medium containing 10% FBS and 1% penicillin streptomycin. After 7 days, the cultured nerve segments were collected and total protein was obtained for the western blot analysis of TRPV4.

Walking track analysis Walking track analysis was performed as previously reported (61). The value of SFI was calculated using the formula below:

$$\text{Sciatic Functional Index (SFI)} = 118.9\left(\frac{\text{ETS} - \text{NTS}}{\text{NTS}}\right) - 51.2\left(\frac{\text{EPL} - \text{NPL}}{\text{NPL}}\right) - 7.5$$

ETS: toe spread (the distance from the first to the fifth toe) of the ipsilateral hind paw; NTS: toe spread of the contralateral hind paw; EPL: paw length (the distance from the heel to the third toe) of the ipsilateral hind paw; NTS: paw length of the contralateral hind paw. In the presence of a toe contracture, the print length was measured as the paw length plus the length from the proximal knuckle to the end of the toe (63).

Electron microscopy Tissue preparation and imaging were performed as previously described (64). Image analysis was performed with Image J software. The g-ratio was calculated based on the diameter of the axon divided by the diameter of the axon including the myelin. The diameter was calculated by the measured perimeter divided by π.

Statistics Three independent experiments were performed. Data are presented as the mean ± SEM. Statistical analysis was performed with Origin Pro8 (RRID: SCR_014212; OriginLab, Haverhill, MA, USA). Significant changes were identified using a two-tailed *t-test*, at 95% confidence interval, with $p < 0.05$ considered as statistically significant (p values: *< 0.05, ** < 0.01).

Results

To clarify the expression and function of TRPV4 in SCs, I isolated and purified SCs from the sciatic nerves of postnatal and adult mice. Both TRPV4 mRNA and protein were detected in the purified SCs by RT-PCR and western blot analyses, which indicates that TRPV4 is expressed in cultured SCs. In addition, TRPV4-mediated responses to 1.0 μM GSK1016790A, a TRPV4 selective agonist, were observed using both calcium-imaging and whole-cell patch-clamp methods. These demonstrate the functional expression of TRPV4 in cultured SCs.

Furthermore, TRPV4 was found to be expressed in sciatic nerves in vivo by western blot. However, I did not observe any differences in the expression levels of the key myelin structural proteins such as myelin-associated glycoprotein (MAG), myelin protein zero (P0) or myelin basic protein (MBP) between WT and TRPV4KO mice by immunostaining and western blot analysis, suggesting that TRPV4 is not involved in normal myelin development in mice. However, after sciatic nerve cut injury, TRPV4 expression gradually increased with sciatic nerve demyelination, even under conditions without macrophages which are known to express TRPV4. This suggests that the

increase in TRPV4 after sciatic nerve injury is mainly derived from SCs. Furthermore, I confirmed that TRPV4 is expressed in unmyelinating SCs, but not in myelinating SCs by double immunostaining of TRPV4 with glial fibrillary acidic protein (GFAP) or MBP in teased sciatic nerves, and that unmyelinating SCs were increased after nerve injury. These results suggest why TRPV4 was increased after injury. I next examined whether the increased TRPV4 is active under physiological conditions. I measured the temperature-evoked increase in intracellular calcium concentrations using a calcium-imaging method in cultured SCs. TRPV4-dependent intracellular calcium increases were clearly observed in the unmyelinating SCs under body temperature, which suggests that TRPV4 is constitutively active in SCs at normal body temperature.

To determine whether the recovery process from sciatic nerve cut-induced Wallerian degeneration is affected by the increase in TRPV4 expression, I created a sciatic nerve cut injury model in both WT and TRPV4KO mice. Interestingly, western blot analysis showed significantly higher levels of P0, MAG and MBP proteins in TRPV4KO mice compared with WT mice 7 days after injury. In addition, I examined the function of sciatic nerves of these mice by walking track analysis 2 months after injury. Sciatic functional index (SFI) values were significantly smaller in TRPV4KO mice than in WT mice, indicating that the absence of TRPV4 impairs the functional recovery of sciatic nerves after injury. To further evaluate the regeneration of sciatic nerves, the structure of the distal stumps from these mice was analyzed using an electron microscope (EM). I found the reformed myelin was significantly thinner in TRPV4KO mice than in WT mice at 2 months after sciatic nerve injury. However, Walking track analysis and EM analysis revealed that the sciatic nerve function and the remyelination were similarly recovered by 6 months both in between WT and TRPV4KO mice after injury. these results demonstrate that the lack of TRPV4 delayed sciatic nerve remyelination and functional recovery following sciatic nerve cut-induced Wallerian degeneration.

Discussion

TRPV4 is mainly expressed in unmyelinating SCs. Following sciatic nerve cut injury, SCs are gradually demyelinated, resulting in an increase in TRPV4. The increased TRPV4 is activated under physiological conditions and plays a pivotal role in this process. Lack of TRPV4 leads to a large accumulation of myelin proteins at the injury site, which could inhibit remyelination. However, the reason for the greater amount of myelin structural proteins in TRPV4KO mice is still unknown. One

possibility is that high levels of these structural proteins in TRPV4KO mice result from a deficiency in myelin breakdown or poor clearance of myelin debris by SCs or macrophages, which interferes with new myelin formation. Another possibility is that TRPV4 may be involved in maintaining the appropriate levels of myelin structural proteins during SC differentiation after injury, which is important for sciatic nerve remyelination. To fully understand the details of this mechanism, further investigations are required.

Thus, I conclude that TRPV4 enhances SC remyelination after sciatic nerve cut injury. My experiments used a sciatic nerve cut injury model, which mimics the neurotmesis in peripheral nerves, in which axons, connective sheaths and basal lamina tubes are interrupted. The model is the most serious nerve injury in nerve damage schemes in rodents, and it takes more than 6 months to recover, although the functional recovery is generally poor and the reformed axon and myelin are thinner than normal condition. However, it is impossible for human to be completely recovered, even though some surgical repair may help. Our studies suggest that our body temperature contributes to the natural recovery of the peripheral nerve injury by activating TRPV4, thus TRPV4 may be an attractive pharmacological target for therapeutic intervention after peripheral nerve injury.