Elevated TRPV4 Levels Contribute to Endothelial Damage and Scarring in Experimental Spinal Cord Injury

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Currently, the role of transient receptor potential vanilloid type 4 (TRPV4), a nonselective cation channel in the pathology of spinal cord injury (SCI), is not recognized. Herein, we report the expression and contribution of TRPV4 in the pathology of scarring and endothelial and secondary damage after SCI. TRPV4 expression increased during the inflammatory phase in female rats after SCI and was expressed primarily by cells at endothelial-microglial junctions. Two-photon microscopy of intracellular-free Ca^{2+} levels revealed a biphasic increase at similar time points after SCI. Expression of TRPV4 at the injury epicenter, but not intracellular-free Ca^{2+} , progressively increases with the severity of the injury. Activation of TRPV4 with specific agonist altered the organization of endothelial cells, affected tight junctions in the hCMEC/D3 BBB cell line *in vitro*, and increases the scarring in rat spinal cord as well as induced endothelial damage. By contrast, suppression of TRPV4 with a specific antagonist or in female *Trpv4* KO mouse attenuated inflammatory cytokines and chemokines, prevented the degradation of tight junction proteins, and preserve blood–spinal cord barrier integrity, thereby attenuate the scarring after SCI. Likewise, secondary damage was reduced, and behavioral outcomes were improved in *Trpv4* KO mice after SCI. These results suggest that increased TRPV4 expression disrupts endothelial cell organization during the early inflammatory phase of SCI, resulting in tissue damage, vascular destabilization, blood–spinal cord barrier breakdown, and scarring. Thus, TRPV4 inhibition/knock-down represents a promising therapeutic strategy to stabilize/protect endothelial cells, attenuate nociception and secondary damage, and reduce scarring after SCI.

Key words: blood–spinal cord barrier; endothelial cell; scarring; spinal cord injury; TRPV4; two-photon microscopy

Significance Statement

TRPV4, a calcium-permeable nonselective cation channel, is widely expressed in both excitable and nonexcitable cells. Spinal cord injury (SCI) majorly caused by trauma/accidents is associated with changes in osmolarity, mechanical injury, and shear stress. After SCI, TRPV4 was increased and were found to be linked with the severity of injury at the epicenter at the time points that were reported to be critical for repair/treatment. Activation of TRPV4 was damaging to endothelial cells that form the blood–spinal cord barrier and thus contributes to scarring (glial and fibrotic). Importantly, inhibition/knockdown of TRPV4 prevented these effects. Thus, the manipulation of TRPV4 signaling might lead to new therapeutic strategies or combinatorial therapies to protect endothelial cells and enhance repair after SCI.

Introduction

The pathology of spinal cord injury (SCI) is multifaceted, encompassing the initial tissue disruption from the primary trauma and

secondary injury progression, involving inflammation, the death of endothelial cells (ECs), neurons, and glia, extracellular matrix remodeling, scarring, and cavitation (Liu et al., 1997; Ling and

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Liu, 2007). Vascular pathology can be observed minutes after SCI, initiated by the death of ECs, which, along with pericytes, astrocytes, and neurons, comprise the microvasculature under normal conditions (Whetstone et al., 2003; Casella et al., 2006). The death of ECs continues throughout the acute phase of injury (Benton et al., 2008), predominantly at the injury epicenter, causing substantial hemorrhage and disturbance of vascular autoregulation that subsequently contributes to the death of neurons and glial cells (Casella et al., 2002, 2006). Microvessels in the spinal cord support the neural parenchyma not only by supplying blood and oxygen but also by continuously secreting neurotrophins, such as BDNF and angiopoietins, which support regenerating spinal cord tissue (Guo et al., 2008). SCI therapies targeting EC preservation have shown remarkable potential (Fassbender et al., 2011; Kumar et al., 2018c); however, therapeutic endothelial protection/stabilization within penumbral microvasculature remains largely unexplored because of a lack of understanding of the key molecular pathways triggered after SCI.

Most mammalian tissues, including the peripheral nervous system and the CNS (Montell et al., 2002; Ramsey et al., 2006), express transient receptor potential vanilloid type 4 (TRPV4), a calcium-permeable nonselective cation channel (Liedtke et al., 2000; Strotmann et al., 2000) that is currently recognized as a polymodal ionotropic receptor (Kumar et al., 2018a). TRPV4 channels are activated by a wide range of stimuli (Liedtke et al., 2000; Strotmann et al., 2000; Willette et al., 2008; Kumar et al., 2018a), as well as intracellular signaling pathways (Alessandri-Haber et al., 2004, 2006; Grant et al., 2007; Zhao et al., 2014). The abundant presence of ion channels in the plasma membrane of ECs suggests their functional role (Nilius and Droogmans, 2001). Ion channels in ECs control several functions, including intracellular Ca^{2+} signals, production and release of many vasoactive factors, regulates macromolecules and also involved in controlling intercellular permeability, EC proliferation, and angiogenesis (Nilius and Droogmans, 2001). Reports suggest that increase in TRPV4 activity disintegrated the cell junctions of blood–CSF barrier (Narita et al., 2015). However, TRPV4 expression and its role following SCI have not been characterized.

Here, we show that TRPV4 expression is associated with the severity of injury at the epicenter after SCI. We demonstrate that TRPV4 upregulation and activation disrupt EC organization after SCI and that ECs are better preserved in the absence of TRPV4. Furthermore, the deletion of TRPV4 signaling significantly impacts SCI-induced inflammatory cascades, blood–spinal cord barrier (BSCB) integrity, degree of scarring, and spontaneous locomotor recovery after SCI. Notably, inhibition or deficiency of TRPV4 was beneficial for endothelial factors, such as BDNF, neurotrophins, and angiopoietin, in the CNS after SCI.

Materials and Methods

Subjects and surgical procedures

A total of 211 female Sprague Dawley rats (220–240 g), for this study, were purchased from Orient Bio. Mice heterozygous ($B6.129X1-Trpv4^{tm1Msz}$) for *Trpv4* deficiency (Suzuki et al., 2003) were purchased from RIKEN BioResource Centre and intercrossed to generate *Trpv4* KO mice. The genotype was examined using PCR with primer sequence as given in Table 1. WT (C57BL/6J) were purchased from Orient Bio served as controls. Thirty-eight female WT and 22 female KO mouse were used in the study. Animals were housed in a facility with 55%–65% humidity at $24 \pm 2^\circ\text{C}$ with a 12 h light/dark cycle and free access to food and water. All animal experiments were performed as per the approved guidelines by the Institutional Animal Care and Use Committee of CHA University (IACUC160073) and Principles of laboratory

Table 1. Primer sequences for the genes of interest used in the current study

Oligo name	Reverse/forward	Sequence (5' → 3')
TRPV4 (rat)	Forward	CCACCCAGTGACAACAAG
	Reverse	GGAGCTTTGGGGCTCTGT
Occludin (rat)	Forward	ATCTAGAGCTGGAGCAACG
	Reverse	GTCAAGGCTCCAGACAAAG
PACSLN3	Forward	AAGGCCCTGAGAGAGGAC
	Reverse	CTCCAGAACTGCCACTA
IL-6 (rat)	Forward	TGATGGATGCTTCCAACTG
	Reverse	GAGCATTGGAAGTTGGGGTA
TNF- α (rat)	Forward	ACTGAACCTCGGGGTGATTG
	Reverse	GCCTGGTGGTTGCTACGAC
HO-1 (rat)	Forward	GTCAAGCACAGGTGACAGA
	Reverse	CTGCAGCTCTCAACACAG
Neuropilin-1 (rat)	Forward	CATAGTGGGCTCGGACTGA
	Reverse	GGGTCCAGCTGAGGCAC
ANG-1 (rat)	Forward	TTAGATTGGAAGGGCCACA
	Reverse	ATGCGCCTTATGCTAACAG
ANG-2 (rat)	Forward	CCCACTCTGAGCTTCACATC
	Reverse	CATAGGAGGAACTGTTACC
Nucleophosmin (rat)	Forward	CCGATGACTGACAGGA
	Reverse	GCAACTATTACAGAAATGAAGACAGAA
Arachidonate 15 lipoxygenase (rat)	Forward	GAAGCTGCTACGACCTGTC
	Reverse	CCAAGGTATCTGACACATCC
Aquaporin-9 (rat)	Forward	CCTTCTGAGAAGGACGAGCC
	Reverse	CTTGAACCACTCCATCTTCC
KCNJ10 (rat)	Forward	GGAGGAGATCTCTGGGGTT
	Reverse	CCACTGGGAGATGCCACTTT
iNOS (rat)	Forward	CTTTGCCACGACGAGAC
	Reverse	TCATTGTACTCTGAGGGCTGAC
GAPDH (rat)	Forward	CAACTCCCTCAAGATTGTACAGAA
	Reverse	GGCATGGACTGTGGTCATGA
Occludin (mouse)	Forward	CCTCCAATGGCAAGTGAAT
	Reverse	CTCCCCACCTGCTGTAGT
VEGF (mouse)	Forward	CGCAAGAAATCCCGGTTTAA
	Reverse	CAAAATGCTTCTCCGCTC TGA
IL-1 β (mouse)	Forward	TTGTGGCTGTGGAGAAGCTGT
	Reverse	AACGTACACACACAGCAGGTT
IL-6 (mouse)	Forward	GCTACCAATGGATATAATCAGGA
	Reverse	CCAGGTAGCTATGGTACTCCAGAA
TNF- α (mouse)	Forward	AGCAAAACCAAGTGGAGGA
	Reverse	GCTGGCACCAGTGTGGTTGT
Mac-1 (mouse)	Forward	CCTTGTCTCTTTGATGACG
	Reverse	GTGATGACAACTAGGATCTT
CCL-2 (mouse)	Forward	GAAGGAATGGGTCCAGACAT
	Reverse	ACGGGTCACTTCACATTC
CCL-3 (mouse)	Forward	TTTGAAACACAGCAGCTTTGCTCC
	Reverse	TCAGGCATTACGTTCCAGTGAGT
GAPDH (mouse)	Forward	CCATTTGTCTACGGGACGA
	Reverse	GGGTTCCTATAAATACGAGTGC
Genotyping		
<i>trpv4</i> exon 4R	Forward	TGTTCCGGGTGGTTGGCCAGGATAT
<i>trpv4</i> neo	Forward	GCTGCATACGCTTGATCCGGCTAC
<i>trpv4</i> exon 4R	Reverse	GCTGAACCAAGGACACTTGCATAG

PACSLN3, Protein kinase C and casein kinase substrate in neurons 3; HO-1, heme oxygenase; KCNJ10, ATP-sensitive inward rectifier potassium channel 10; iNOS, inducible nitric oxide; CCL-2, C-C motif chemokine ligand-2; CCL-3, C-C motif chemokine ligand-3; Mac-1, macrophage-1 antigen.

animal care (National Research Council, 2011). Animals were anesthetized for laminectomies exposing the ~T10 segment of the spinal cord as previously reported (Kumar et al., 2018b). The vertebral column was stabilized and supported by Allis clamps at T8, and T12 spinous processes as previously described (Kumar et al., 2018b,c) (see Fig. 1A). A metal impounder (rats: 20 g/5 min [mild injury], 35 g/5 min [moderate injury], or 50 g/5 min [severe injury]; mice: 20 g/1 min) was then gently applied to T10 dura, resulting in weight compression SCI. Animals were anesthetized for laminectomies exposing the ~T10 segment of the spinal cord,

and a transverse cut was made to create a right hemisection injury. The surgical site was closed after SCI, by suturing the muscle and fascia using silk suture followed by suturing the skin. Povidone-iodine was applied externally to the surgical site, and animals were kept on a heating pad to maintain body temperature until they recovered from anesthesia, and then 5 ml (rats) or 0.5 ml (mouse) of 0.9% sterile saline-injected subcutaneously. Manual bladder expression was performed twice daily (morning and evening) until a bladder reflex was established.

Cell culture, drugs, and treatments

Blood–brain barrier (BBB) hCMEC/D3 cell line (Millipore) and human umbilical vascular endothelial cells (HUVECs; ATCC) were used to understand the biological role of TRPV4 activation or inhibition in ECs. hCMEC/D3 and HUVECs were cultured in fully supplemented endothelial growth medium (PromoCell, Human Centered Science) as per the manufacturer's instructions. GSK1016790A and RN-1734 were purchased from Sigma Millipore. GSK1016790A and RN-1734 were dissolved in DMSO and then used at the functional concentration of 1 and 10 μ M, respectively, for *in vitro* studies. RN-1734 was dissolved in 5% DMSO, 5% *N,N*-dimethylacetamide and remaining saline and administered intraperitoneally at the dose of 5 mg/kg 1 h after SCI. Rats were killed at 8 h after injury (HPI-8; calcium imaging) or 1 d postinjury (DPI-1; biochemical parameters) after vehicle or RN-1734 treatment. GSK1016790A was first dissolved in DMSO, and then serial dilution was performed to get the final concentration of 50 pmol [0.3% DMSO in aCSF (Tocris Bioscience)]; subsequently, 10 μ l was injected at $\sim$ T10 level of spinal cord with the help of Legato 130 Syringe Pump (KD Scientific).

Behavioral assessment

Hindlimb locomotor score. Hindlimb locomotor function was evaluated using the open-field Basso Mouse Scale (BMS) locomotor test (Basso et al., 2006) on 1, 3, 7, 14, 21, and 28 d following injury in WT ($n = 10$) and TRPV4 KO ($n = 10$) mice. The scoring ranged from 0 points (no ankle movement) to 9 points (complete functional recovery). The animal's hindlimb motor functions were evaluated by two experienced investigators who were blinded to treatment group.

Test for nociception. Nociception in mice was assessed using the hotplate method at pre-SCI (basal), 1, 3, 7, 14, 21, and 28 d after SCI. Briefly, mice ($n = 6$ /group) were placed on the surface of the hotplate (25.4 $\times$ 25.4 cm; Ugo Basile) heated to 54°C, which was surrounded by a transparent Plexiglas chamber (see Fig. 6A). The latency to respond was measured with either a hindpaw lick or flick, with a cutoff threshold of 20 s to prevent tissue injury. Mice were removed immediately from the hotplate after a response. A total of three readings were used to determine the average reaction time.

qRT-PCR

qRT-PCR was performed at respective time points using a SYBR Green Master Mix, and the mRNA detection was analyzed using an ABI StepOne Real-time PCR System (Applied Biosystems) (Kumar et al., 2018c). Primer sequences for the genes of interest used in the current study were as given in Table 1: Typical profile times were the initial step, 95°C for 10 min followed by a second step at 95°C for 15 s, and 60°C for 30 s for 40 cycles with a melting curve analysis. The target mRNA level was normalized with the level of the GAPDH and compared with the control. Data were analyzed using the $\Delta\Delta$ CT method.

Western blot analysis

hCMEC/D3 cells were collected after 24 h of drug treatment. Spinal cord tissues were collected at DPI-1 and DPI-28 and washed with PBS, placed at 4°C, and homogenized in lysis buffer (PRO-PREP, iNtRON Biotechnology) and centrifuged at 14,000 rpm at 4°C for 15 min. The supernatant was collected for determination of protein concentration using Bio-Rad DC Protein Assay. Protein concentration was determined by VersaMax microplate reader. Equal amounts of protein (40 μ g) were separated electrophoretically by 10% SDS-PAGE electrophoresis, and the resolved proteins were transferred to PVDF membranes (#162-0177, Bio-Rad). The membranes were then incubated for 1 h with 5% nonfat skim milk prepared in TBS buffer to block nonspecific binding. The membranes were then incubated overnight in cold room with primary

Table 2. Primary antibody information used for immunoblot analysis

Primary antibody	Dilution	Producer company	Code number
TRPV4	1:200	Alomone Labs, Israel	ACC-034
ANG-1	1:10,000	Abcam, UK	ab183701
NF	1:10,000	Abcam, UK	ab8135
ZO-1	1:1000	Cell Signaling Technology, USA	8193
ZO-2	1:1000	Cell Signaling Technology, USA	2847
Claudin-1	1:1000	Cell Signaling Technology, USA	13255
CD2AP	1:1000	Cell Signaling Technology, USA	2135
Afadin	1:1000	Cell Signaling Technology, USA	13531
IL-6	1:1000	Abcam, UK	ab6672
Occludin	1:500	Invitrogen, USA	33-1500
CD-31	1:500	Abcam, UK	ab28364
AKT	1:1000	Cell Signaling Technology, USA	9271
JNK	1:1000	Cell Signaling Technology, USA	4668
ERK	1:1000	Cell Signaling Technology, USA	4377
Caspase-3	1:1000	Cell Signaling Technology, USA	96625
Iba-1	1:1000	FUJIFILM Wako, Japan	019-19741
Connexin-43	1:5000	Abcam, UK	ab11369
GFAP	1:10,000	Abcam, UK	Ab16997
Actin	1:10,000	ABM, Canada	G043

Table 3. Primary antibody information used for immunohistochemistry analysis

Primary antibody	Dilution	Producer company	Code number
Anti-TRPV4	1:500	Alomone Labs, Israel	ACC-034
Anti-RECA-1	1:100	Abcam, UK	ab22492
Anti-NF	1:1000	Abcam, UK	ab8135
Anti-Iba-1	1:200	FUJIFILM Wako, Japan	019-19741
Anti-laminin	1:50	Sigma	L9393
Anti-GFAP	1:500	Abcam, UK	ab10062
Anti-ZO-1	1:50	Invitrogen	40-2300
Anti-occludin	1:50	Invitrogen	33-1500
Anti-collagen-IV	1:500	Abcam, UK	ab6586
Anti-nitrotyrosine	1:100	Abcam, UK	ab183391
Anti-CD-68	1:200	Abcam, UK	ab955
Anti-TGF- β 1	1:100	Abcam, UK	ab25121
Anti-CD-206	1:500	Abcam, UK	ab64693
Anti-BDNF	1:300	Alomone Labs, Israel	AGP-021
Anti-NT-3	1:200	Alomone Labs, Israel	ANT-003
Anti-vWF	1:50	Millipore	AB7356
Anti-ANG-1	1:200	Abcam, UK	ab133425
Anti-NG-2	1:500	Abcam, UK	ab129051
Anti- α -SMA	1:200	Abcam, UK	ab21027
Anti-connexin-43	1:500	Abcam, UK	ab11369
Anti-Tuj-1	1:1000	Abcam, UK	ab18207
Anti-growth cone	1:3	Abcam, UK	ab7762
Anti-NeuN	1:1000	Abcam, UK	ab177487

antibodies as given in Table 2. After 1 h incubation at room temperature with corresponding secondary antibodies, the blots were visualized with enhanced chemiluminescence (GE Healthcare), using the LAS 4000 bio-molecular imager (GE Healthcare). The immunoblots were quantified using ImageJ software (Fiji).

Immunohistochemistry and Immunofluorescence

After SCI at T10, animals were anesthetized and perfused with 0.9% saline, followed by 4% PFA for tissue fixation at several time points as per the time points and treatments. The spinal cord at compression site was retrieved and immersed in 4% PFA for 1 d, and then fixed in paraffin, sectioned at 5 μ m, dewaxed, and stained with primary antibodies overnight at 4°C followed by secondary antibody as given in Tables 3 and 4. Following PBS washing, DAPI (1:500) was incubated for 10 min. Sections were mounted in ACRYMOUNT mounting media (StatLab) and examined using a fluorescence microscope (Carl Zeiss or Leica Microsystems). Image quantification for immunofluorescence signals was performed

Table 4. Secondary antibody information used for immunohistochemistry analysis

Secondary antibody	Dilution	Producer company	Code number
Goat anti-rabbit AlexaFluor-488	1:200	Abcam, UK	ab150077
Goat anti-rabbit AlexaFluor-568	1:200	Abcam, UK	ab175471
Goat anti-rabbit AlexaFluor-647	1:200	Abcam, UK	ab150083
Goat anti-mouse AlexaFluor-568	1:200	Abcam, UK	ab175473
Goat anti-mouse AlexaFluor-488	1:200	Abcam, UK	ab150117
Goat anti-guinea pig AlexaFluor-488	1:200	Abcam, UK	ab150185
Chicken-anti-goat 647	1:200	Invitrogen, USA	A-21469

using Zen 3.0 [Blue edition (Carl Zeiss)]. Equal area (nm^2) was selected, channel intensities were measured, and fluorescence intensity mean value (IMV) was obtained. The fluorescence IMV was plotted as mean $\pm$ SEM in GraphPad Prism (version 5.01, GraphPad Software).

Immunocytochemistry

For immunofluorescence microscopy, third passage hCMEC/D3 BBB cell line and HUVECs (0.5×10^5 cells/well) were cultured on a sterile rat tail collagen-I (Sigma Millipore)-coated coverslip in 24-well plates. Immunocytochemistry was performed as per previously published protocol (Kumar et al., 2018b). Cells were incubated overnight at 4°C with antibodies directed against TRPV4 (1:100, Alomone Labs) and CD-31 (1:100, Abcam). Secondary antibodies (1:500) were goat anti-rabbit AlexaFluor-488 and goat anti-mouse AlexaFluor-488 (Abcam). Following DAPI incubation for 10 min, coverslips were mounted and examined using a fluorescence microscope (Carl Zeiss).

Calcium imaging using two-photon fluorescence microscopy

We evaluated the $[\text{Ca}^{2+}]_i$ inside the live rat or mouse spinal cord tissue after the injury as per the previously reported method (Kim et al., 2017). Briefly, a thin sectional tissue slice was extracted from the injured area following SCI at 3 and 8 HPI and at 1, 3, 5, 7, 14, 21, and 28 DPI for effective two-photon microscopy (TPM) imaging. Consequently, we incubated the spinal cord sectional slice with $10 \mu\text{M}$ Ca^{2+} sensing unit $\text{SCa1-I}_{\text{REF}}$ for 40 min at 37°C, and ratiometric TPM images were acquired at depths of 90–210 μm for visualization of the overall Ca^{2+} distribution (Kim et al., 2017). The TPM images of $\text{SCa1-I}_{\text{REF}}$ -labeled tissues were acquired with spectral confocal and multiphoton microscopes (Leica Microsystems, TCS SP8 MP) with $\times 10$ dry and $\times 40$ oil objectives, numerical aperture (NA) = 0.40 and 1.30 as previously described (Kim et al., 2017). Ratiometric image processing and analysis were performed using MetaMorph software (Molecular Devices).

Evaluation of blood–spinal cord barrier permeability

To establish the role of TRPV4 in BSCB disruption, we examined samples from sham, injured WT, and injured TRPV4-KO mouse prepared DPI-1. The BSCB permeability was investigated with Evans blue dye extravasation according to a previous report (Kumar et al., 2018b). Briefly, at DPI-1 after SCI, 500 μl of 2% Evans blue dye (Sigma Millipore) solution prepared in saline and administered intraperitoneally. Mouse were anesthetized 3 h after Evans blue dye injection and killed by intracardiac perfusion with saline. Spinal cord segment was collected and homogenized in 50% trichloroacetic acid solution. Homogenate samples were centrifuged at $10,000 \times g$ for 10 min, and supernatant was collected. Fluorescence intensity of Evans blue was quantified using a spectrophotometer at excitation wavelength of 620 nm and emission wavelength of 680 nm as previously described (Kumar et al., 2018b).

TUNEL assay

Paraffin sections from TRPV4 KO and WT mouse were examined for apoptotic cells at DPI-1 using an *in situ* death detection kit (Roche Diagnostics) as per the manufacturer's information. The images were examined using an Olympus microscope (U-TVO.63XC).

Statistical analyses

Statistical analysis was performed using GraphPad Prism (version 5.01, GraphPad Software). Data are presented as mean $\pm$ SEM from the indicated number of experiments. The *in vivo* PCR data were analyzed using one-way ANOVA, followed by Tukey's test. The BMS scores and hotplate

data were analyzed statistically with two-way ANOVA followed by the Bonferroni test. *p* values < 0.05 were considered statistically significant. The respective statistical analysis used to analyze the data is mentioned in the figure legends.

Results

TRPV4 expression increases during the early phase of SCI

We first characterized the time course of TRPV4 expression in rats at the epicenter of the SCI at 3 and 8 HPI and at 1, 3, 5, 7, 14, 21, and 28 DPI (35 g for 5 min) (Fig. 1A). TRPV4 expression was low in the uninjured spinal cord (sham condition), localized mainly to ECs, blood vessels, and neurons, and substantially increased during the early phase after SCI (Fig. 1C,F; Fig. 1-1A, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f1-1>). The integrity of the vascular endothelium was assessed by immunostaining for rat EC antigen (RECA) (Fig. 1D; Fig. 1-1B, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f1-1>) and angiopoietin-1 [(ANG-1), primarily expressed by pericytes] (Fig. 1-1E,F, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f1-1>). Neurofilament (NF), a marker of the neuronal cytoskeleton (Fig. 1D; Fig. 1-1B,E,F, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f1-1>) was used to evaluate the neuronal damage after injury. ECs were lost in the injury epicenter as early as HPI-3, with some recovery by DPI-5, whereas NF immunoreactivity gradually declined with time after injury (Fig. 1D; Fig. 1-1B,D,E,F, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f1-1>). Intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$) were quantitated *in situ* at the same time points by using TPM (Fig. 1B), which revealed a biphasic response to SCI (Fig. 1E,G; Fig. 1-1C, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f1-1>). SCI induced a secondary injury cascade via the production of inflammatory mediators, such as IL-6. IL-6, as assessed by qRT-PCR, showed a transient increase at early time points from HPI-3 to DPI-1 after injury (Fig. 1H). qRT-PCR analysis showed the altered expression of protein kinase C and casein kinase substrate in neurons 3 (Pacsin-3), a member of the family of proteins involved in synaptic vesicular membrane trafficking that also strongly inhibits TRPV4 expression (D'hoedt et al., 2008) (Fig. 1I).

Injury-dependent TRPV4 expression at the epicenter of SCI

Next, we also investigated whether TRPV4 followed an injury-dependent expression. Rats were subjected to mild (20 g/5 min), moderate (35 g/5 min), and severe (50 g/5 min) SCI (Fig. 2A–C). TRPV4 expression increased with the severity of the injury at the epicenter area compared with the sham group (Fig. 2D,I; Fig. 2-1A, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f2-1>). The expression of TRPV4 was also increased at the rostral area in mild and severe SCI (Fig. 2D). However, the increase in $[\text{Ca}^{2+}]_i$ measured by TPM at HPI-8 did not correspond to the severity of the injury (Fig. 2J,H). Pacsin-3 expression was declined with an increase in the injury (Fig. 2F). Assessments of inflammation (IL-6 [Fig. 2E]; HO-1, ANG-2, and TNF- α [Fig. 2-1B,D,I, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f2-1>]), BSCB integrity, endothelial and vascular damage (occludin [Fig. 2G]; RECA [Fig. 2L]; neuropilin, ANG-2, and ANG-1 [Fig. 2-1C,D,J, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f2-1>]), and neuronal damage (NF [Fig. 2L]), were congruent with the injury severity. TRPV4 immunoreactivity assessed at DPI-1 colocalized with that for the microglial marker Iba-1 and for endothelial-specific marker CD-31, indicating that ECs and microglia express TRPV4 after SCI (Fig. 2K). Furthermore, the expression of TRPV4, IL-6, and

Figure 1. TRPV4 expression increased during the inflammatory/acute phase of SCI. **A**, Schematic showing SCI instrument and method. **B**, Schematic illustration showing TPM imaging after SCI. Representative immunohistochemistry images performed on longitudinal sections at epicenter of the damage for TRPV4 (**C**), and NF and RECA-1 (**D**) at 3 h, 8 h, and 1, 3, 5, and 7 d after moderate static compression (35 g/5 min) SCI (Carl Zeiss microscope, 3 fields/slide, $n = 3$ /time point). SCI rat sectional slice labeled with 10 μ M Sca1-IREF for 40 min. *In situ* Ca^{2+} levels were determined in the transverse spinal cord using TPM at similar time points after SCI at the epicenter of the damage. **E**, Two-photon excited fluorescence was collected using 750 nm excitation and emission windows at 400–430 nm (Ch1) and 500–600 nm (Ch2). Total RNA was prepared from the epicenter of the damage collected 3 h, 8 h, and 1, 3, 5, 7, 14, 21, and 28 d after SCI to determine the expression of TRPV4 (**F**), IL-6 (**H**), and Pacsin-3 (**I**) ($n = 4$ –6/time point, performed in triplicates). **G**, Quantification of *in situ* Ca^{2+} levels. GAPDH was used as internal controls for qRT-PCR. Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with sham group. $^{\$}p < 0.05$, $^{SS}p < 0.001$ compared with HPI-3. $^{\#}p < 0.05$ compared with HPI-8. $^{\textcircled{p}}p < 0.05$, $^{\textcircled{p}p}p < 0.01$, $^{\textcircled{p}pp}p < 0.001$ compared with DPI-1. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ compared with DPI-3. $^{\textcircled{p}}p < 0.05$, $^{\textcircled{p}p}p < 0.01$ compared with DPI-5. $^{\textcircled{p}}p < 0.05$, $^{\textcircled{p}pp}p < 0.001$ compared with DPI-7. $^{\textcircled{p}}p < 0.05$, $^{\textcircled{p}pp}p < 0.001$ compared with DPI-21 by one-way ANOVA Tukey's post test.

Pacsin-3 was altered by hemisection of the spinal cord; however, the increase in TRPV4 was to a lesser extent than that with compression SCI (Fig. 2M–O). Together, these results suggest that TRPV4 expression and related parameters are associated with the severity of compression injury.

Activation of TRPV4 promotes endothelial damage, whereas inhibition is protective

To evaluate the effect of TRPV4 activation on ECs and the BSCB, we incubated hCMEC/D3 BBB cells, which have basal expression of TRPV4, for 24 h with a TRPV4 agonist (1 μ M GSK1016790A [GSK]) or antagonist (10 μ M RN-1734), which results in increased or decreased TRPV4 expression, respectively (Fig. 3A,B). The CD-31 immunoreactivity to assess the integrity/status of the ECs was reduced by TRPV4 activation; this reduction was mitigated by pretreatment with the antagonist (Fig. 3A,C). Accordingly, TRPV4 agonist or antagonist results in increased or decreased TRPV4 expression, respectively, in HUVECs (Fig. 3D,E). Similar to hCMEC/D3 BBB cells, endothelial integrity/status, as measured by von Willebrand factor (vWF) immunoreactivity, was altered by TRPV4 activation, and this was prevented by the TRPV4 antagonist (Fig. 3D,F). The expression of TJ markers zonula occludens-1 (ZO-1), ZO-2, and claudin-1 was attenuated by GSK (1 μ M), with a prominent effect observed for ZO-2 and claudin-1; however, the TRPV4 antagonist RN-1734 (10 μ M) or RN-1734 treatment before GSK either maintained or increased the expression of the TJ markers (Fig. 3-1A, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f3-1>). To assess the effect of TRPV4 activation *in vivo*, GSK (50 μ M) was injected

directly into the spinal cords of rats. We examined samples from uninjured sham and GSK-treated animals after 28 d as well as from vehicle and GSK-treated animals at DPI-28. SCI causes centralized fibrotic scars surrounded by a reactive glial scar at the epicenter. Damaged vessels immunoreactive for laminin, a component of the basal lamina, were readily identified at DPI-28 (Fig. 3G). A single injection of GSK changed/damaged the morphology of laminin structures (J- or T-shaped under normal conditions) and RECA-stained vessels (Fig. 3G,J). The basal lamina also comprises collagen IV, and the normal uniform distribution was altered by GSK injection; a dense collagen IV meshwork was observed at DPI-28 in animals treated with vehicle or GSK (Fig. 3-1N,O, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f3-1>). Immunoreactivity for ANG-1, a CNS endothelium neurotrophin, was decreased after GSK treatment (Fig. 3-1N,O, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f3-1>).

We also evaluated the effect of TRPV4 activation alone or with injury at day 28. Interestingly, GSK injections only produced an increase in microglial cells (as determined by Iba-1 immunoreactivity) but not in reactive astrocytes (as determined by GFAP immunoreactivity) (Fig. 3H,K). Immunoreactivity for the axonal marker NF revealed significant loss with GSK injection and extensive damage in injured spinal cords compared with that in the sham group (Fig. 3I,L). Additionally, blood infiltration following BSCB disruption after SCI initiates a secondary injury cascade via the production of inflammatory mediators, such as TNF- α , IL-6, and inducible nitric oxide synthase. As the activation of TRPV4 appeared to damage ECs, we examined the effect

Figure 2. TRPV4 expression is linked with injury severity and inflammation. Schematic showing method for injury-dependent expression and impounder of different weight (**A,B**). H&E staining was performed on the longitudinal section after mild (20 g/5 min), moderate (35 g/5 min), and severe (50 g/5 min) injury (**C**). Total RNA was prepared from spinal cord tissues at the rostral, epicenter, and caudal of the damage collected at 1 d after SCI (mild, moderate, and severe compression) to determine the expression of TRPV4 (**D**), IL-6 (**E**), Pacsin-3 (**F**), and occludin (**G**). RNA expression was determined at the epicenter at 1 d after SCI (mild, moderate, and severe) ($n = 2$ or 3 /group performed in triplicates). TRPV4 (**I**) and NF and RECA-1 (**L**) IHC was performed after mild (20 g), moderate (35 g), and severe (50 g) injury (Carl Zeiss microscope, 3 fields/slide, $n = 3$ /time point). *In situ* Ca^{2+} levels were determined using TPM at 8 h after SCI (mild, moderate, and severe) at the epicenter of the damage (**J**). Quantification of *in situ* Ca^{2+} levels (**H**). Colocalization of TRPV4 with CD-31, an endothelial marker, and Iba-1, a microglial marker (**K**). Total RNA was prepared from spinal cord tissues after spinal cord hemisection to determine the expression of TRPV4 (**M**), IL-6 (**N**), and Pacsin-3 (**O**) ($n = 4$ or 5 /group). GAPDH was used as internal controls for qRT-PCR. Data are mean $\pm$ SEM performed in triplicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with sham group. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ compared with 20 g injury group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with 35 g injury group by one-way ANOVA Tukey's post test.

of TRPV4 inhibition on BSCB integrity, inflammation, and Ca^{2+} levels during the early phase after SCI, when there is peak TRPV4 expression. Samples from sham, injured vehicle-treated, and injured RN-1734-treated (5 mg/kg, i.p.) animals were analyzed at DPI-1. Treatment with RN-1734 significantly inhibited TRPV4 expression attenuated SCI-induced inflammatory cytokines and prevented the loss of the TJ proteins occludin and ZO-1 after injury (Fig. 3*M,N,P,Q,S–W*; Fig. 3-1*H–M*, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f3-1>). In addition, treatment with RN-1734 significantly reduced the SCI-induced calcium release as determined by Ca^{2+} imaging at HPI-8 (Fig. 3*O,R*).

TRPV4 KO mice have less endothelial damage and inflammation

On the basis of the above-described results, we hypothesized that the ablation of TRPV4 signaling would protect ECs and preserve BSCB integrity. To test this, we induced SCI (20 g/1 min) at $\sim$ T10

in TRPV4 KO and WT mice at DPI-1 (Fig. 4*A*). Evans blue extravasations consistent with an increase in barrier permeability were observed in WT mice but not in TRPV4 KO mice (Fig. 4*B,C*). The loss of TJ proteins, such as occludin, ZO-1, ZO-2, and claudin-1, did not occur in TRPV4 KO mice (Fig. 4*D,L,Q*; Fig. 4-1*A,C–F*, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f4-1>), and ECs were preserved, as determined by higher CD-31 levels (Fig. 4-1*A,G*, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f4-1>), accompanied by an attenuation of the increase in VEGF expression after injury (Fig. 4*E*). The $\sim$ 100 kDa band for TRPV4 was barely detectable in the KO mice compared with the WT after injury (Fig. 4-1*A,B*, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f4-1>). Pro-inflammatory cytokine and chemokine production increased in WT mice after SCI compared with that in sham controls, and this was significantly attenuated in TRPV4 KO mice (Fig. 4*F–K*).

We performed TUNEL assays to assess apoptosis after SCI. At DPI-1, apoptotic cells were present at the epicenter in both WT

Figure 3. TRPV4 activation causes endothelial remodeling/damage, whereas TRPV4 inhibition protects SCI-induced endothelial damage. TRPV4 and CD-31 (**A**) immunocytochemistry was performed on fixed hCMEC/D3 BBB cell line as described in Materials and Methods. Quantification of TRPV4 and CD-31 fluorescence (**B,C**). TRPV4 and vWF immunocytochemistry was performed on fixed HUVECs (**D**) and its quantification (**E,F**). GSK1016790A (50 pmol, 10 μ l) was injected into the spinal cord of the rats as mentioned in Materials and Methods. Samples from sham, GSK1016790A, vehicle (injury), or GSK1016790A (injury) were prepared 28 d after injection/injury. Representative images of laminin (magenta) and RECA-1 (**G**; green), GFAP (**H**; green), Iba-1 (**H**; red), and NFs (**I**; red). **J–L**, Bar charts represent the fluorescence IMV for corresponding protein as per randomly selected field area at the injury epicenter (3 fields/slide, $n = 3$ /group). Samples from sham or injured untreated (injury) or after RN-1734 (5 mg/kg) treatment were prepared 1 d after moderate injury (35 g/5 min). Representative transverse section of TRPV4 (**M**) and ZO-1 (**N**) (3 fields/slide, $n = 3$) and fluorescence IMV for corresponding protein (**P,Q**). Total RNA from sham, vehicle (injury), or RN-1734-treated (5 mg/kg, i.p.) samples was prepared DPI-1 after injury. RT-PCR results are showing relative expression levels of TRPV4 (**S**), Pacsin3 (**T**), inducible nitric oxide synthase (**U**), IL-6 (**V**), and occludin (**W**). *In situ* Ca^{2+} levels were determined in the transverse spinal cord using TPM at 8 h after SCI at the epicenter of the damage (**O**). Quantification of *in situ* Ca^{2+} levels (**R**). Data are mean $\pm$ SEM ($n = 2$ or 3/group performed in triplicates). * $p < 0.05$, ** $p < 0.01$, compared with sham group. $^{\$}p < 0.05$, $^{\$ \$}p < 0.01$, $^{\$ \$ \$}p < 0.001$ compared with GSK group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with sham group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared with injury group (**P–W**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with sham group. $^{\$}p < 0.05$, $^{\$ \$}p < 0.01$, $^{\$ \$ \$}p < 0.001$ compared with GSK group (**J–L**) by one-way ANOVA Tukey's post test.

and KO mice; however, the percentages of TUNEL-positive cells and the amount of caspase-3 were much lower in TRPV4 KO mice than in WT mice (Fig. 4*L,M*; Fig. 4-1*A,L*, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f4-1>). ERK, JNK, and AKT expression was not significantly different between TRPV4 KO and WT mice, whereas KO mice showed a reduction in p38 expression (Fig. 4-1*A,H–K*, available at <https://doi.org/10.1523/JNEUROSCI.2035-19.2020.f4-1>). TPM imaging at HPI-8 revealed that SCI-induced $[\text{Ca}^{2+}]_i$ was inhibited in TRPV4 KO mice (Fig. 4*N,O*).

Reduced scarring in TRPV4 KO mice after SCI

To further assess the endothelial protective effects of TRPV4 ablation on scarring, we assessed scarring (glial and fibrotic) 28 d after SCI. Whereas the lesions of WT animals contained large

amounts of laminin and collagen IV, hallmarks of the fibrotic scars thought to hinder axon regeneration, the amounts in lesions of TRPV4 KO mice were much lower (Fig. 5*A,B,G,I*). The expression of GFAP indicative of glial formation was higher at DPI-28 in WT than in TRPV4 KO mice, whereas the expression of NF, a marker for neuronal cytoskeleton, was higher at DPI-28 in TRPV4 KO than in WT mice, suggesting that KO mice had attenuated glial scarring and better neuronal protection (Fig. 5*C,K,M,N,Q*). Similarly, immunostaining for Iba-1 to detect resident microglia and infiltrating monocyte-derived macrophages showed significantly reduced expression in TRPV4 KO mice compared with that in WT mice (Fig. 5*D,H,M,O*). By contrast, the expression of CD-206, a marker for M2 macrophages, was increased in TRPV4 KO mice (Fig. 5*E,J*). Immunoreactivity for TGF- β , a key mediator of fibrotic scarring, and CD-68, a

Figure 4. Reduced endothelial damage and inflammation after SCI in TRPV4 KO mice. Representative images of H&E staining at DPI-1 after SCI (**A**). Representative whole spinal cords show Evans blue dye extravasation of the spinal cord 1 d after SCI (**B**) and its quantification (**C**). Total RNA from sham (black bar), wild (red bar), or TRPV4 KO (blue bar) samples was prepared DPI-1 after the injury as described in Materials and Methods. RT-PCR results show relative expression levels of occludin (**D**), VEGF (**E**), IL-1β (**F**), IL-6 (**G**), TNF-α (**H**), macrophage-1 antigen (Mac-1) (**I**), chemokine (C-C motif) ligand 2 (CCL-2) (**J**), and chemokine (C-C motif) ligand 3 (CCL-3) (**K**) after injury ($n = 2$ or 3/group performed in triplicates). GAPDH was used as internal controls for qRT-PCR. TUNEL assay was performed at DPI-1 (**L**). Quantification of TUNEL-positive cells (**M**). SCI-induced mouse sectional slice labeled with 10 μM Sca1-IREF for 40 min. *In situ* Ca²⁺ levels were determined in the longitudinal spinal cord using TPM at 8 h after SCI at the epicenter of the damage. Two-photon excited fluorescence was collected using 750 nm excitation and emission windows at 400–430 nm (Ch1) and 500–600 nm and its quantification (Ch2) (**N,O**). Representative images for ZO-1 (magenta) and occludin (**Q**; green) and fluorescence IMV for corresponding protein (**P**). ### $p < 0.001$ compared with sham group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with WT-injury group (**D–K,O**) by one-way ANOVA Tukey's post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs WT-injury group (**C,M,P**) by unpaired *t* test.

marker for activated macrophages, was significantly lower in TRPV4 KO mice than in WT mice (Fig. 5*F,L*). The expression of connexin-43, an abundant gap junction protein in the CNS, was markedly higher in WT mice than in TRPV4 KO mice, whereas CD-31, which makes up a large portion of EC intercellular junctions, was higher in TRPV4 KO mice (Fig. 5*M,P,R*).

TRPV4 KO mice show improved functional recovery and neuroprotection

To evaluate the functional consequences of TRPV4 ablation, we assessed gross locomotor function in TRPV4 KO and WT mice using the BMS to assess open field hindlimb locomotion (Basso et

al., 2006). TRPV4 KO mouse spinal cord was better preserved compared with WT as determined by H&E staining (Fig. 6*A*). Mice developed paraplegia after SCI, corresponding to a low BMS score at DPI-1–3, but demonstrated evidence of modest improvement as early as DPI-7 (Fig. 6*B*). The BMS scores over 4 weeks ranged from 0.30 ± 0.48 to 4.7 ± 2.0 for WT mice and from 0.50 ± 0.71 to 7.00 ± 2.00 for TRPV4 KO mice, with TRPV4 ablation associated with faster recovery and better motor coordination in comparison with that for WT (Fig. 6*B*). The performance of sham control mice remained unchanged throughout the testing period (data not shown). As nociception is also affected after SCI, we evaluated the effect of TRPV4 dele-

Figure 5. TRPV4 KO mice exhibit less fibrotic and glial scarring after SCI. Samples from WT (injury) or TRPV4 KO (injury) were prepared at DPI-28 as described in Materials and Methods. Representative merges images (longitudinal) for Collagen IV (**A**; white), laminin (**B**; magenta), GFAP (**C**; green), NF (**C**; magenta), Iba-1 (**D**; magenta), CD-206 (**E**; green), CD-68 (**F**; green), and TGF- β 1 (**F**; magenta) at DPI-28. Bar charts represent the fluorescence IMV for Collagen IV (**G**), Iba-1 (**H**), laminin (**I**), CD-206 (**J**), GFAP and NF (**K**), and TGF- β 1 and CD-68 (**L**) as per randomly selected field area at the injury epicenter (3 fields/slide, $n = 3$ /group). **M**, Western blots of NFs, Iba-1, Connexin-43, GFAP, and CD-31 expression at 28 d after injury. Quantification of immunoblot for NF (**N**), Iba-1 (**O**), Connexin-43 (**P**), GFAP (**Q**), and CD-31 (**R**) was performed using ImageJ. Actin was used as internal controls for Western blot ($n = 2$ or 3/group). Data are mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs WT-injury group by unpaired t test.

tion on SCI-induced pain using the hotplate test (Fig. 6C). After SCI, animals exhibited thermal hypersensitivity (decrease in reaction time) on days 1, 3, 7, and 14 but not at 21 and 28 d (Fig. 6D). This hypersensitivity was significantly attenuated at DPI-1, DPI-3, and DPI-7 in TRPV4 KO mice. Immunoreactivity for ANG-1, BDNF, and neurotrophin 3 (NT-3) neurotrophic factors, which are primary mediators of axonal and neuronal plasticity and regeneration after SCI, was also greater in TRPV4 KO mice than in WT mice after SCI (Fig. 6E, F). We also assessed the effects of TRPV4 deletion on neuroprotection and angiogenesis after SCI. Immunohistochemistry of microvessels in spinal cord sections revealed that expression of vWF (Fig. 6G) and neural/glial antigen2 (NG-2) and α -smooth muscle actin (α -SMA) (Fig. 6H) was higher in TRPV4 KO mice after injury than in WT mice. Accordingly, immunoreactivity for growth cones was more evident in axons in TRPV4 KO mice than in WT mice (Fig. 6J), which was accompanied by greater immunoreactivity for neuronal markers TuJ1 and NeuN, indicating better neuronal protection in TRPV4 KO mice (Fig. 6I, J). These results suggest that abrogation of TRPV4 signaling is neuroprotective during SCI.

Discussion

In the present study, we establish that TRPV4, a nonselective cation channel expressed by astrocytes and neurons (Ryskamp et al., 2014; Shibasaki et al., 2014), is a crucial determinant of endothelial disruption and the pathology after SCI. We show that SCI

induces the expression of TRPV4 at earlier time points; however, the free $[Ca^{2+}]_i$ level follows a biphasic increase. However, there may be various mechanisms by which SCI produces these effects. (1) The increase in TRPV4 expression may be induced by mechanical stimulation as a result of cell swelling, as TRPV4 is also a volume-sensitive mechanosensor (Nilius et al., 2001; Shi et al., 2013). (2) Most Ca^{2+} -permeable channels show some feedback regulation by Ca^{2+} to prevent deleteriously significant increases in $[Ca^{2+}]_i$ or to shape the time course of channel activity, and experimental evidence shows that TRPV4 is tightly regulated by $[Ca^{2+}]_i$ (Strotmann et al., 2003). (3) TRPV4 is highly sensitive to changes in extracellular osmolarity, as reductions in the extracellular osmolarity result in increases in $[Ca^{2+}]_i$ and membrane currents and osmolarities >300 mosmol/L decrease both $[Ca^{2+}]_i$ and currents (Strotmann et al., 2000). (4) Ca^{2+} is involved in both the decaying phase and the activation phase of TRPV4 (Plant and Strotmann, 2006). Traumatic SCI can result in contusion, compression, and stretch injury. Both compression and contusion models simulate the biomechanics and neuropathology of human injury (Sharif-Alhoseini et al., 2017). In the current study, we used clinically relevant SCI via controlled mechanical compression (Yu et al., 2013; Ropper et al., 2015). The advantage of the current method is that delivered force is precisely known at the time of application compared with other compression models. One limitation of the current methods is the variability in

Figure 6. TRPV4 KO mice display reduced hyperalgesia, improved functional recovery, enhanced neuroprotection, and endothelial preservation after SCI. **A**, Representative images of H&E staining at DPI-28 after SCI. **B**, Functional recovery was assessed in open-field testing by using the 9-point BMS locomotor test at 1, 3, 7, 14, 21, and 28 d after SCI ($n = 10$ /group). **C**, Nociception was evaluated using hotplate test. **D**, SCI-induced hypersensitivity (decrease in reaction latency time) was assessed at pre-SCI (basal), 1, 3, 7, 14, 21, and 28 d after SCI ($n = 6$ /group). Samples from WT (injury) or TRPV4 KO (injury) were prepared at DPI-28 as described in Materials and Methods. Representative sections of angiopoietin-1 (ANG-1, white), NT-3 (red), and BDNF (green) in WT (**E**) and KO (**F**). Representative section of vWF (**G**; green), neural/glial antigen NG-2 (**H**; green), α -SMA (**H**; red), Connexin-43 (**I**; green), Tuj-1 (**I**; magenta), growth cone (green; **J**), and NeuN (magenta, **J**). Bar charts represent the IMV (fluorescence) for corresponding protein as per randomly selected field area at the injury epicenter (3 fields/slide, $n = 3$ /group). Data are mean $\pm$ SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT-injury group.

stabilizing and supporting the spinal column using Allis clamps at T8 and T12 spinous processes. Ideally, the spinal cord should be parallel to the impounder during compression to avoid inconsistent parenchymal injury. Second, the compression with this method is only applied to dorsally to the spinal cord.

We also found that the expression of TRPV4 at the injury epicenter, but not $[Ca^{2+}]_i$, progressively increases with the severity of the injury. TRPV4 does not have an apparent Ca^{2+} -binding site, such as an EF-hand, suggesting that Ca^{2+} does not bind directly to the channel. Cells expressing TRPV4 often display spontaneous channel activity, resulting in an elevated basal $[Ca^{2+}]_i$ (Plant and Strotmann, 2006), but there are also reports showing no changes in $[Ca^{2+}]_i$ in cells expressing TRPV4 (Strotmann et al., 2000). Nonetheless, in the present study, $[Ca^{2+}]_i$ was increased after injury, regardless of the severity. Thus, our findings suggest that TRPV4 expression might be useful for predicting the severity of the CNS injury. We also observed progressive damage to the vascular endothelium and neurons (via RECA and NF immunoreactivity, respectively). Interestingly, RECA immunoreactivity was low at DPI-1 when the expression of TRPV4 was high and matched the initial progressive damage to the endothelium, whereas axons showed progressive damage after SCI. These observations suggest that TRPV4 is increased and might promote the EC detachment following SCI.

Previous reports suggest that the expression of the TJ proteins occludin and ZO-1 is downregulated after moderate compression injury (Kumar et al., 2018c), suggesting a disruption of the BSCB. In the present study, the expression of occludin decreased

while that of inflammatory cytokines (IL-6 and others) increased, suggesting the direct involvement of TRPV4 in BSCB disruption and inflammation. The upregulation of TRPV4 after SCI was also associated with a decrease in Pacsin-3, a synaptic vesicular membrane trafficking protein that inhibits TRPV4 basal expression (D'hoedt et al., 2008), suggesting that the upregulation of TRPV4 has functional relevance. TRPV4 is expressed by vascular ECs in rodents (Watanabe et al., 2002; Zhang et al., 2009) and humans (Sullivan et al., 2012) and has also been identified in vascular smooth muscle cells (Senadheera et al., 2013) and in the astrocytic endfoot processes that wrap around blood vessels in the CNS (Benfenati et al., 2011). TRPV4 activation within the astrocytic endfeet contributes to neurovascular coupling via Ca^{2+} entry (Dunn et al., 2011).

Adhesion, tight, and gap junctions connect the ECs lining the vessel walls (Cerutti and Ridley, 2017). In the current study, pharmacological activation of TRPV4 decreased the expression of TJ markers (ZO-1, ZO-2, and claudin-1) and remodel adhesion junctions (via afadin and endothelial actin-binding protein CD2-associated protein). *In vivo* application of a TRPV4 agonist led to EC damage and basal lamina deposition, which could diminish angiogenesis and promote cystic cavity formation, and enhanced EC damage after SCI. After SCI in rodents and humans, scarring occurs at the lesion site, comprising glial cells and laminin and fibronectin (Ruschel et al., 2015), which impedes axonal regeneration (Silver and Miller, 2004; Leal-Filho, 2011). However, the glial scars in animals receiving injections of the TRPV4 agonist were predominantly composed of microglia, which supports the

notion that ion channels are also involved in regulating microglial functions (Färber and Kettenmann, 2005).

The activation of microglia after CNS injury is linked with neuroinflammation and impairments of the BBB/BSCB. TRPV4 activation also stimulates neuropeptide release from afferent nerves and induces neurogenic inflammation (Vergnolle et al., 2010). Whereas TRPV4 is activated by changes in osmolarity, which leads to increased IL-1 β and IL-6 in intervertebral discs and is suggestive of increased release of proinflammatory cytokines (Walter et al., 2016), we found that inhibition of TRPV4 (via RN-1734) attenuated inflammation, reduced free [Ca²⁺]_i levels, and preserved the BSCB.

The functional properties of TJ components and the selective leakiness of the CNS TJs are well studied (Bazzoni and Dejana, 2004; Abbott et al., 2010). Damage to these proteins leads to BSCB instability and increased permeability, which promotes inflammatory infiltration in the CNS, the major determinant of secondary injury (Noble and Wrathall, 1989). The temporal and spatial increase of secondary damage after SCI has been attributed to the augmented expression of sulfonylurea receptor 1-regulated NC_{Ca-ATP} channels, such as Trpm4, by ECs (Gerzanic et al., 2009). In the present study, we found that ECs in animals with TRPV4 deficiency were protected from damage after SCI, suggesting that the activation of TRPV4 after injury might directly affect EC survival. A possible explanation is that TRPV4-mediated entry of Ca²⁺ into ECs regulates the production of nitric oxide and responses to inflammatory signals by changing the barrier properties (Tiruppathi et al., 2002). For example, knockdown of TRPV4 in adipocytes is inhibitory for an array of cytokines and chemokines in adipose tissues, suggesting that TRPV4 positively controls proinflammatory genes (Ye et al., 2012). Consistent with this, we found that TRPV4 KO mice had reduced inflammation and synthesis of chemokines and cytokines after SCI as well as enhanced EC integrity, reduced permeability, and reduced apoptosis. After SCI, the expression of ANG-1 was reduced. ANG-1 lowers vascular leakage/inflammation and expedites angiogenesis (Lee et al., 2009), by strengthening related endothelial molecules and regulating interendothelial adhesion. We found that the reduction in ANG-1 was attenuated in TRPV4 KO mice. Additionally, TRPV4 KO mice maintained the levels of several neurotrophins (BDNF and NT-3), which are linked with EC survival (Donovan et al., 2000), as well as levels of vWF, NG-2, and SMA. Notably, the detachment of ECs, which persists throughout the acute injury phase (Koyanagi et al., 1993), is an indirect contributor to neuronal and glial cell death (Casella et al., 2002).

Inflammation is well established in the pathology of SCI, and contributes to fibrosis scarring in part via TGF- β signaling. The increase in TGF- β /CD-68 expression after SCI was attenuated in TRPV4 KO mice. We also observed an SCI-induced increase in astrocytes, microglia, extracellular matrix proteins, and gap junctions. The persistence of these glial cells indicates a proinflammatory environment, linked with increased neurotoxicity and impaired wound healing. However, these increases were attenuated in TRPV4 KO mice, suggesting that the increase in TRPV4 observed in WT animals enhanced the scarring process. Indeed, SCI-induced endothelial damage was augmented, and glial scar formation was higher in animals injected with the TRPV4 agonist. Scarring leads to the formation of a sharp lesion border, which impairs neuronal regeneration (Bundesen et al., 2003), by forming a physical barrier preventing regenerating axons from extending across the injury site. A reduction of one or many specific cellular components of the scar can influence the scarring

(Meletis et al., 2008; Barnabé-Heider et al., 2010; Göritz et al., 2011; Zhu et al., 2015). Although TRPV4 also mediates neurotrophic factor-induced neuritogenesis in developing peripheral nerves (Jang et al., 2012), activation of this channel in the CNS via intracerebroventricular injection of the agonist induces neuronal apoptosis (Jie et al., 2015, 2016). Accordingly, we observed reduced immunoreactivity for NF, a cytoskeleton protein prominently expressed in larger axons, in animals injected with GSK, suggesting that TRPV4 activation affects large axons after SCI.

Finally, the sparing or regeneration of the vasculature and endothelial functions after injury correlates with improved functional outcomes (Kaneko et al., 2006). The above-described reduction in glial scarring and inflammation and enhanced expression of neuronal markers in KO mice suggest that TRPV4 contributes to the pathology of, and impaired recovery from, SCI. Accordingly, we observed reduced hyperalgesia toward heat and faster locomotor recovery in TRPV4 KO mice after SCI. The observed functional recovery might be attributed to reduced reactive gliosis. However, TRPV4 deficiency does not affect escape latency in response to heat in the absence of hyperalgesia (Liedtke and Friedman, 2003; Suzuki et al., 2003; Todaka et al., 2004), although an increase in tail withdrawal latency to moderately hot temperatures was reported (Lee et al., 2005). Other studies suggest that the channel contributes to the sensation of noxious mechanical stimuli (Liedtke and Friedman, 2003; Suzuki et al., 2003). Together, these data suggest that TRPV4 contributes to nociception only in the setting of inflammation or nerve injury (Alessandri-Haber et al., 2008).

In conclusion, TRPV4 expression is rapidly induced following SCI and in accordance with the severity of the injury. Its expression is linked to endothelial damage, inflammation, and apoptosis, thereby increasing secondary injury. Importantly, inhibition/knockdown of TRPV4 prevented these effects. Thus, the manipulation of TRPV4 signaling might lead to new therapeutic strategies or combinatorial therapies to protect ECs and enhance repair after SCI.

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