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Supplemental Information

Nuclear Factor-kappaB Gates $Na_v1.7$ Channels in DRG Neurons via Protein-Protein Interaction

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Supplemental Information

Figure. S1

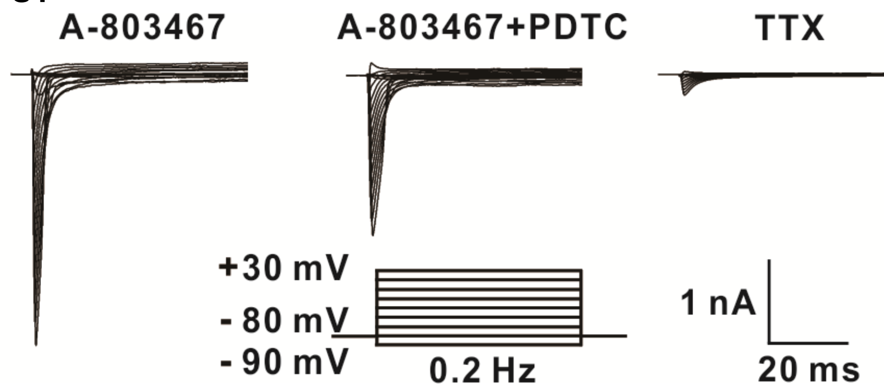

Figure S1. Isolation of TTX-S currents. Related to Figure 2. TTX-S currents were initially identified by their relatively fast activation and inactivation kinetics and recorded in the presence of TTX-R channel blocker A-803467 (1 μM). The identification was confirmed by adding 300 nM TTX at the end of the recordings. Only the current recordings that can be reduced by 90% or more with 300 nM TTX were used for further analysis.

Figure. S2

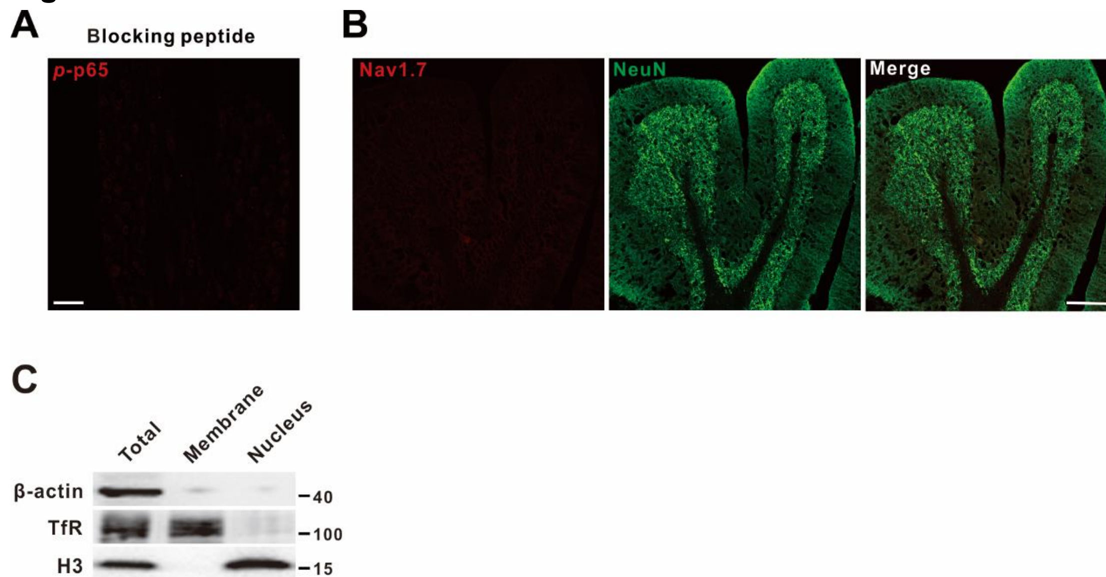

Figure S2. Identification of specificity of anti-*p*-p65 and anti-Na$_v$1.7, and the efficacy of extraction kit for isolation of membrane, cytoplasm and nuclear proteins used in this work. Related to Figure 1, Figure 3 and Figure 6. (A) The specificity of anti-*p*-p65 was identified by pre-incubation with *p*-p65 (s311) blocking peptides provided by manufacturer. Scale bars: 100 μm. (B) Anti-Na$_v$1.7 detect no signal in cerebellum which does not express Na$_v$1.7. Scale bars: 200 μm. (C) The expression of β-actin (marker of cytoplasmic protein), transferrin receptor (TfR) (marker of membrane protein) and histone H3 (marker of nucleoprotein) in the sample of total, membrane and nuclear extracts.

Transparent Methods

Animals

Male Sprague-Dawley rats (80 to 250 g) were purchased from the Institute of Experimental Animals of Sun Yat-sen University. The rats were individually housed in separate cages in a temperature-controlled (24 ± 1°C) and humidity-controlled (50-60%) room, under a 12/12-h light/dark cycle and with ad libitum access to sterile water and standard laboratory chow. All animal experimental procedures were approved by the local Animal Care Committee and were carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines (Zimmermann, 1983). Animals were randomly assigned to different experimental or control conditions.

Preparation of pain models

Chemotherapy-induced peripheral neuropathy was induced by intraperitoneal injection of vincristine sulfate (0.1 mg/kg daily) for 10 consecutive days (Xu et al., 2017). Control animals received an equivalent volume of saline. Lumbar 5 spinal nerve ligation (L5-SNL) was done following the procedures described previously (Xie et al., 2017). Briefly, animals were anesthetized with halothane (2%), the left L5 spinal nerve was isolated adjacent to the vertebral column, and tightly ligated with a 6–0 silk sutures. While in sham-operated rat the L5 spinal nerve was identically exposed but not ligated.

Drug administration and behavioral test

Intrathecal injection of PDTC or vehicle was performed according to our previously described method (Zhang et al., 2018). In brief, a polyethylene-10 catheter was inserted into the rat's subarachnoid space through L5 and L6 intervertebral space and the tip of the catheter was located at the L5 spinal segmental level.

Mechanical sensitivity was assessed using von Frey hairs with the up-down method as described previously (Chaplan et al., 1994). Briefly, each rat was placed in a transparent Plexiglas testing chamber positioned on a wire mesh floor. A series of calibrated von Frey hairs with different bending forces were applied from below through the mesh floor to the sciatic innervation area of the hind paws for about 6-8 s with a 5 min interval between stimuli. Brisk withdrawal or licking of the paw in response to the stimulus was considered as positive response. The operator who performed the behavioral tests was blinded to all treatments.

DRG neuron preparation

Primary DRG neuron cultures were prepared from young Sprague-Dawley male rats (80~120 g body weight) as previously described (Xie et al., 2017; Zhang et al., 2018). In brief, L4-6 DRGs were freed from their connective tissue sheaths and broken into pieces with a pair of sclerotic scissors in DMEM/F12 medium (Gibco, USA) under low temperature. DRG neurons were plated on glass cover slips coated with Poly-L-Lysine (Sigma, USA) in a humidified atmosphere (5% CO_2, 37°C) following enzymatic and mechanical dissociation. The cells were used for electrophysiological recordings approximately 4 h to 24 h after plating.

Electrophysiology recordings

Whole-cell patch clamp recordings of Na^+ currents in DRG neurons were performed using an EPC-10 amplifier and the PULSE program (HEKA Electronics, Lambrecht, Germany) as

previously described (Xie et al., 2017). Currents were recorded with glass pipettes (3–5 MΩ resistance) fabricated from borosilicate glass capillaries using a Sutter P-97 puller (Sutter Instruments, Novato, CA). The currents were filtered at 10 kHz and sampled at 50 kHz. Voltage errors were minimized by using 80–90% series resistance compensation. The neurons with a leak current of > 500 pA or a series resistance of > 10 MOhm were excluded. For voltage clamp experiments, the extracellular solution contained (in mM): 30 NaCl, 20 TEA-Cl, 90 choline-Cl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose, and 0.1 $CdCl_2$ (adjusted to pH 7.3 with Tris base). The pipette solution contained (in mM): 135 CsF, 10 NaCl, 10 HEPES, 5 EGTA, and 2 Na_2ATP (adjusted to pH 7.2 with CsOH). For recording TTX-S currents 1 μM A-803467 was included to block the TTX-R Na^+ currents. TTX-S Na^+ currents were identified initially by their relatively fast activation and inactivation kinetics and was confirmed by adding 300 nM TTX at the end of the recordings. Only the current recordings that can be reduced by 90% or more with 300 nM TTX were used for further analysis (Figure S1). And for recording TTX-R Na^+ currents 300 nM TTX was included to block the TTX-S channels (Tanaka et al., 2015). $Na_v1.7$ current was isolated from total Na^+ currents by subtraction of the ProTxII-resistant Na^+ currents from total current using a previously published subtraction protocol (Li et al., 2018; Schmalhofer et al., 2008). In this work, we randomly recorded 153 neurons, including 33 large size of neurons (> 35 μm), 84 medium size of neurons (25-35 μm) and 36 small size of neurons (< 25 μm).
To study the effects of different chemicals on Na_v channels, Na^+ current was elicited by a depolarization potential (from -90 mV to -10 mV, 100 ms). The amplitude of currents evoked by the *n*th impulse was normalized to the current evoked by the first impulse. For calculation of I-V curves, Na^+ current was evoked from a holding potential of -90 mV and then depolarized from -80 mV to +60 mV at 5 mV steps. Current density was calculated by normalizing maximal peak currents with cell capacitance.
To investigate the mechanisms by which *p*-p65 may regulate $Na_v1.7$ channels, The effects of PDTC on activation, inactivation and recovery of $Na_v1.7$ channels were determined in HEK293 cells that express Nav1.7. For building activation curves, the cell was clamped at a holding potential of -90 mV and a prepulse voltage to -120 mV for 200 ms was applied. $Na_v1.7$ current was elicited by a stepped depolarization test voltage pulse from -80 mV to 100 mV for 50 ms. To build steady state fast inactivation curves, the cell was clamped at a holding potential of -90 mV, a stepped prepulse from -120 mV to 40 mV with 5 mV increments for 1000 ms was applied, and the $Na_v1.7$ current was recorded at a voltage of 0 mV. Time constants for recovery from the inactivation of $Na_v1.7$ channel was measured with a double-pulse protocol. A first pulse (P1) for 250 ms to -10 mV caused inactivation, and $Na_v1.7$ current evoked by the test pulse (P2) to -10 mV after variable intervals was compared with $I_{Na,P1}$ of the same episode.
The activation or inactivation conductance variables of I_{Na} were determined with normalized currents. Current activation and inactivation were fitted by the Boltzmann distribution: $y=1/\{1+\exp[(V_m-V_{0.5})/S]$, where V_m is the membrane potential, $V_{0.5}$ is the activation or inactivation voltage mid-point, and S is the slope factor. The relation of $1/\tau_{block}$ against the concentration is described by the linear function: $1/\tau_{block}=k[D]+l$, where $1/\tau_{block}$ is the time constant of development of block, and k and l are the apparent rate constants for association and dissociation of the drug.

Western blot

The L4-6 DRGs were dissected and homogenized in cold RIPA buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, and 1 mg/ml leupeptin]. Membrane, cytoplasm and nuclear proteins were isolated with the protein extraction kit (Invent Biotechnologies, SM-005). The isolation efficacy of the kit was identified (Figure S2C). The protein samples were separated via gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The membranes were placed in blocking buffer for 1 h at room temperature and incubated in a primary antibody against *p*-p65 (ser311) (1:100, mouse; Santa Cruz Biotechnology; sc-135769), transferrin receptor (TfR) (1:1000, mouse; Invitrogen; QG215340), $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, $Na_v1.9$ (1:200, rabbit; Alomone Labs; ASC-004 for $Na_v1.3$; ASC-009 for $Na_v1.6$; ASC-008 for $Na_v1.7$; ASC-016 for $Na_v1.8$; ASC-017 for $Na_v1.9$), Histone H3 (1:1000, rabbit; Affinity; AF0863) overnight at 4°C. And then, the membranes were incubated in HRP-conjugated secondary antibody. Enhanced chemiluminescence (ECL) solution (Milipore) was used to detect the immunocomplexes. Each band was quantified with a computer-assisted imaging analysis system (Tanon Gis).

Co-Immunoprecipitation

The dissected DRG tissues were lysed in cold co-IP RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, and 1 mg/ml leupeptin]. The lysate was centrifuged and 5% of the supernatant was used for input sample. The remaining supernatant was precipitated with 10 μg anti-*p*-p65 or anti-$Na_v1.7$ at 4°C overnight and then with protein A/G beads (GE Healthcare) at 4°C for 4 h. The immunoprecipitated sample was denatured and prepared for immunoblotting. Immunoprecipitation was performed with antibodies against $Na_v1.3$, $Na_v1.6$, $Na_v1.7$, $Na_v1.8$, $Na_v1.9$ and *p*-p65.

Immunohistochemistry and structured illumination microscopy

Rats were perfused with 4% paraformaldehyde (PFA). The L4-6 DRGs were dissected and post-fixed in 4% PFA for 1 h. Then the tissues were dehydrated in 30% sucrose and embedded for cryostat sectioning. The cryostat sections were incubated with primary antibodies against *p*-p65 (ser311) (1:50, rabbit; Santa Cruz Biotechnology; sc-33039), $Na_v1.7$ (1:100, mouse; Abcam; ab85015), IB4 (1:50; Sigma; L2895), CGRP (1:200, mouse; Abcam; ab81887), NF200 (1:200, mouse; Sigma; N0142), GFAP (1:400, mouse; Cell signaling technology; 3670) at 4°C overnight, and then incubated in secondary antibodies for 1 h at room temperature. Three-dimensional super-resolution images were captured using a three-dimensional structured illumination microscope with the N-SIM System and an oil immersion objective lens CFI SR (Apochromat TIRF×100, 1.49 numerical aperture, Nikon, Japan), and images were post-processed with Nikon NIS-Elements software. The specificity of the antibody for *p*-p65 (s311) and $Na_v1.7$ was identified in Figures S2A and S2B.

Solutions and chemicals

All solution was adjusted to pH 7.35-7.40 and to osmolality 310 mOsm. Vincristine sulfate (Main Luck Pharmaceuticals Inc.) was dissolved in saline to a concentration of 50 μg/ml immediately before application. Tetrodotoxin (Absin) was dissolved as a stock of 1 mM in acetic acid aqueous solution and diluted to a working concentration of 300 nM. A-803467 (Selleck) was dissolved in DMSO as a stock of 1 mM, diluted to 1 μM with extracellular solution. TNF-α (R&D) were dissolved with in sterile PBS containing 0.1% bovine serum albumin and diluted to work concentration (100 nM). ProTxII (TOCRIS) were dissolved with distilled water and diluted

to work concentration (5 nM) with extracellular solution. ICA121431 (MCE) was dissolved with distilled water and diluted to work concentration (5 μM) with extracellular solution. PDTC (Sigma Aldrich) was diluted to 10 nM in extracellular solution. *P*-p65 antibody (rabbit; Santa Cruz Biotechnology) was diluted to 10 μg/ml in pipette solution.

Data analysis

All data were expressed as mean ± SD, and analyzed with GraphPad Prism 7. Threshold for statistical significance was $P < 0.05$. Although no power analysis was performed, the sample size was determined according to previous publications in pain-associated behavior and molecular studies.

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