SUPPLEMENTAL MATERIAL

Piezo1 is a mechanosensor channel in central nervous system capillaries

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MATERIALS AND METHODS

Animal models

Adult (2–5-month-old) male and female mice were group-housed on a 12-hour light:dark cycle with environmental enrichment and free access to food and water. Animals were euthanized by intraperitoneal injection of sodium pentobarbital (100 mg/kg) followed by rapid decapitation. All experimental protocols used in this study are in accord with institutional guidelines approved by the Institutional Animal Care and Use Committee of the University of Vermont. This study complies with ARRIVE guidelines. Mice used include the previously described *Cdh5*-GCaMP8 transgenic mouse line (22), in which the high signal-to-noise Ca^{2+} indicator GCaMP8 is expressed under the transcriptional control of the endothelial promoter *Cdh5*; wild-type C57BL/6J mice (Jackson Laboratories, USA); TRPV4 knockout (TRPV4$^{-/-}$) mice; and inducible brain endothelial $G\alpha_{q/11}$ knockout (be-$G\alpha_{q/11}$ KO) (52). Inducible, EC-specific Piezo1-knockout (*Piezo1*-EC-KO) mice were generated by crossing mice in which exons 20-23 of the *Piezo1* gene are flanked by loxP sites (*Piezo1*$^{fl/fl}$, Jackson Laboratories, USA) with mice expressing an inducible *Cre*-recombinase under the transcriptional control of *Cdh5* (*Cdh5*-Cre/ERT2). For induction of *Piezo1* knockout, offspring homozygous for floxed *Piezo1* (*Piezo1*$^{fl/fl}$) and expressing *Cre*-recombinase were treated with tamoxifen by providing tamoxifen citrate food (Envigo, TD.130859) for 7 consecutive days (daily tamoxifen intake, ~40 mg/kg), followed by a minimum 7-day washout period before any experimental intervention. Tamoxifen-treated *Piezo1*$^{fl/fl}$ littermates that lacked expression of *Cre*-recombinase (*Piezo1*-EC-Ctrl) were used as controls. Genotyping was performed for all mice to verify that Piezo1 was floxed and confirm the presence (*Piezo1*-EC-KO) or absence (*Piezo1*-EC-Ctrl) of *Cdh5*-Cre prior to tamoxifen treatment. Because specific antibodies for mouse Piezo1 are lacking, we used a functional approach (electrophysiology) to confirm the loss of Piezo1 activity. For electrophysiology experiments, the mice used were: (i) experiments in Figs. 2, 3 and 4: all male C57BL/6J mice; (ii) experiments in Fig. 5c: 2 male + 2 female *Piezo1*-EC-Ctrl mice and 1 male + 2 female *Piezo1*-EC-KO mice; (iii) experiments in Fig. 5d: 1 male + 3 female *Piezo1*-EC-Ctrl mice and 2 male + 2 female *Piezo1*-EC-KO mice; (iv) experiments in Fig. 5e-h: all male C57BL/6J mice; and (v) experiments in Fig. 6: male C57BL/6J mice, male TRPV4$^{-/-}$ mice, 1 male + 2 female be-$G\alpha_{q/11}$ KO mice. For retina imaging experiments, the *Cdh5*-GCaMP8 mice that we used were: (i) experiments in Fig. 1c-d: 3 male + 3 female; (ii) experiments in Fig. 1e: 1 male + 2 female; (iii) experiments in Fig. 7c-d: 2 male + 1 female; (iv) experiments in Fig. 7i-j: 5 male + 2 female; and (v) experiments in Fig. 7k-m: 1 male + 2 female.

Ex vivo Imaging of EC Ca^{2+} in the retina

Cdh5-GCaMP8 mice were anesthetized, and retinas were isolated and pinned down in an *en face* (vitreal side up) orientation, as previously reported (23). Retinal preparations were mounted on a silicone block, washed, and incubated in physiological saline solution (PSS) bubbled with 95% O_2/5% CO_2 for 20 minutes at 37°C; thereafter, the entire retina was continuously perfused with 37°C PSS buffer at 5 mL/min. After cannulating the ophthalmic artery, the retinal vasculature was pressurized via a gravity-fed line attached to a perfusion pressure monitor (PM-4; Living Systems) by moving the pressure column to ~80 mmHg (corresponding to pressure at the ophthalmic artery) and opening a two-way valve. Note that inlet pressure is measured in the cannula tubing at the level of the cannulated ophthalmic artery; thus, the measuring system does not impose a resistance. Rapid removal of blood cells from all retinal vessels confirmed successful cannulation and pressurization. Retinal ECs were imaged using a fluorescence macro zoom imaging system (Olympus MVX10; Olympus). Different inlet pressures (20 and 80 mmHg) were delivered and measured at the ophthalmic artery. Although actual downstream pressures are not precisely known and direct measures of ophthalmic artery pressure are limited, available measurements indicate that the 20 to 80 mmHg pressure values used here bracket the estimated physiological range (23). Thus, the changes applied would be

expected to translate into changes in pressure/flow at the capillary level that are within the physiological range. Note that retinal arterioles in this preparation demonstrate a physiological myogenic response at ophthalmic artery pressures from 40-120 mmHg (23). In a subset of experiments, retinal preparations were imaged using a Revolution confocal system (Andor Technology) mounted on an upright Nikon microscope equipped with a 60× water-dipping objective (numerical aperture [NA] 1.0). Fluorescence was excited with a 488-nm solid-state laser, and emitted fluorescence was collected through a 527.5/49 nm band-pass filter. Glass microinjection pipettes filled with 30 μM Yoda1 were guided to targeted capillary segments using a micromanipulator (Sutter Instruments), and a small volume was pressure-injected using a Picospritzer III (Parker). Representative Ca^{2+} fluorescence images included in the manuscript depict median responses to mechanical or pharmacological stimulation and were selected by eye based on signal-to-noise ratios.

cEC isolation

Single cECs were obtained from mouse retinas or brains by mechanical disruption of two retinal cups or a volume of ~10–15 mm^3 of somatosensory cortical tissue, respectively, using a Dounce homogenizer, as previously described (19, 28). Tissues were homogenized in ice-cold isolation buffer (124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 4 mM glucose), and homogenates were passed through a 62-μm nylon mesh. The filtered vasculature retained on the nylon mesh was eluted using a dissociation solution (55 mM NaCl, 80 mM Na-glutamate, 5.6 mM KCl, 2 mM $MgCl_2$, 4 mM glucose, 10 mM HEPES pH 7.3) containing protease (0.5 mg/mL), elastase (0.5 mg/mL; Worthington, USA) and 100 μM $CaCl_2$, and the resulting suspension was incubated for 23 minutes at 37°C. After adding 0.5 mg/mL collagenase Type I (Worthington, USA) and incubating for 2 minutes, the suspension was filtered, and the enzymes were removed by washing the retained material. Single capillary cells were dispersed by triturating 4–6 times using a glass Pasteur pipette. Isolated cECs are readily distinguished from arteriolar ECs by their different morphology: whereas cECs appear as collapsed forms of the small tubes that exist *in vivo*, arterial ECs, which have a characteristic cobblestone appearance *in vivo*, appear as patches of cells with a roundish shape upon isolation. cECs can further be distinguished from arterial/arteriolar ECs by their lack of functional Ca^{2+}-activated K^+ channel currents, providing an internal functional control for the isolation procedure (19, 28, 63). A few drops of the cEC suspension were added to the recording chamber containing enzyme-free dissociation solution. Cells were used within ~6 hours of dispersion.

Electrophysiology

Single-channel currents were recorded in the cell-attached configuration using a patch-clamp amplifier (Axopatch 200B; Molecular Devices), filtered at 1 kHz, digitized at 5 kHz, and stored for subsequent analysis with Clampfit 10.3 software. The sampling rate was 20 kHz, and the low-pass filter frequency was 1 kHz. The bath solution consisted of 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM glucose, and 2 mM $CaCl_2$ (pH 7.35). Recording pipettes were pulled from borosilicate glass (1.5 mm outer diameter, 1.17 mm inner diameter; Sutter Instruments, USA) using a Narishige puller. Pipettes were fire-polished to a tip resistance of ~4 MΩ. The pipette solution consisted of 3 or 6 mM KCl, 137 or 134 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, 4 mM glucose, and 2 mM $CaCl_2$ (pH 7.35). In some experiments, the pipette and bath solution were supplemented with different pharmacological agents as indicated in the text. Representative current traces in the manuscript depict the median response for each experimental condition or genotype.

Estimation of conductance: The default holding potential was -50 mV in most experiments, and a range of voltages (-30, -50, -70, -90 and -110 mV) was used in a subset of experiments to assess unitary currents (using amplitude histograms) and calculate slope conductance. For

recordings at a single voltage (-50 mV), single-channel conductance was calculated as the reciprocal of resistance, estimated using Ohm's law ($V = i * R$) and assuming a reversal potential of 0 mV.

Mechanical activation of cell-attached patches was achieved by applying a calibrated negative pressure stimulus using a pneumatic transducer (Fluke). In the majority of experiments, -10 mmHg was applied. Pressure stimuli more negative than -15 mmHg typically resulted in instability or complete loss of the seal and were therefore avoided. The minimum recording duration for each cEC was 300 seconds.

Reagents

Yoda1, gadolinium, GSK 1016790A, and GSK 2193874 were purchased from Tocris Bioscience (USA). GsMTx4 was purchased from Alomone Labs. All other chemicals and reagents were obtained from Sigma-Aldrich (USA).

Statistical analysis

Data in figures and text are presented as means ± standard error of the mean (SEM), unless otherwise stated. All experiments were performed in a randomized manner (animals, pharmacological treatments). Statistical tests were performed using GraphPad Prism 9 software. Since most tests of normality are not powered to detect departures from normality when n is small, non-parametric tests were used for analyses. In particular, statistical significance was determined using Kruskal-Wallis test, Mann Whitney test or Wilcoxon test, as specified in figure legends. *P*-values < 0.05 were considered statistically significant. Blinding procedures were not generally used because of the limited number of groups and the difficulty in adapting them during experiments using specific genotypes, although some imaging data presented in Figure 7 were analyzed in a blinded manner. Animals were excluded from analysis in cases where GCaMP8 expression was low in *Cdh5*-GCaMP8 mice (assessed by the experimenter) or in the event that signal-to-noise ratio was low in Ca^{2+}-imaging experiments. Sample size was estimated based on previous larger samples reported in the literature by our group and others (22, 28, 63).

Data Availability

All study data are included in the article and supporting information.

SUPPLEMENTAL FIGURES

Supplemental Figure 1

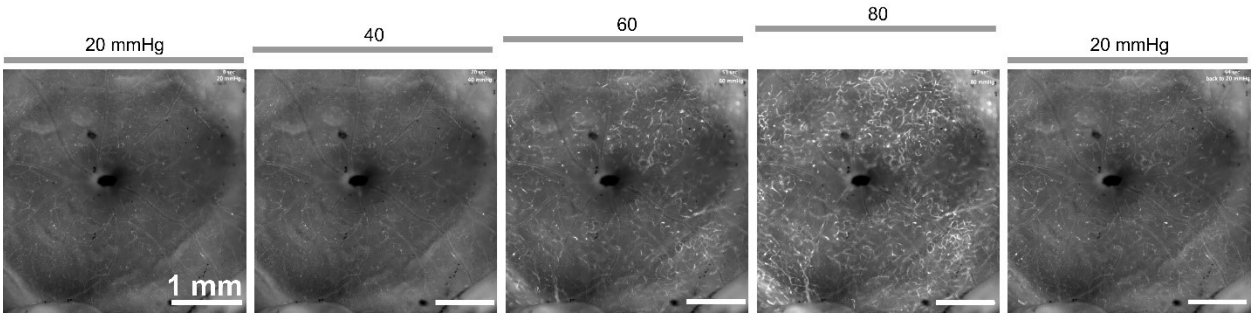

Figure S1. Representative images of the entire retina preparation in response to pressure changes. *En face* retina preparation from a *Cdh5*-GCaMP8 mouse shows EC Ca^{2+} transients in response to elevating intravascular pressure at the cannulated ophthalmic artery from 20 to 40 to 60 to 80 and then back to 20 mmHg.

Supplemental Figure 2

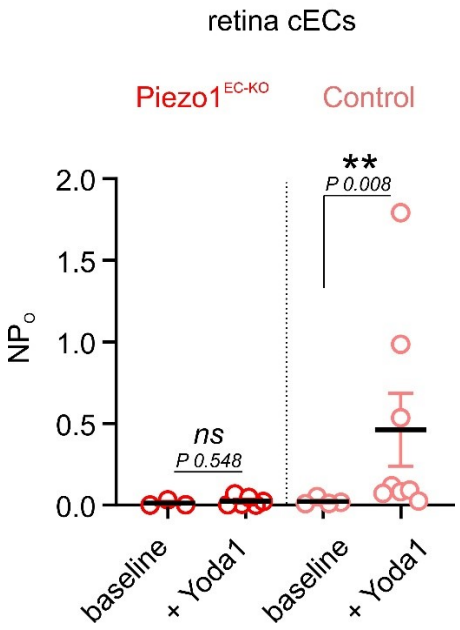

Figure S2. Genetic deletion of Piezo1 abolishes Piezo1 activity in retinal capillaries. Scatter plots of open probability (NP_0) of Yoda1-induced channel openings in Piezo1^{EC-KO} and control cECs. Recordings were performed in the cell-attached mode at a holding potential of -50 mV (Piezo1^{EC-KO}, n = 9 cells from 2 mice; Control, n = 12 cells from 2 mice). Black horizontal lines are mean values, and error bars (pink and red) represent SEM (**P < 0.01, Mann-Whitney test).

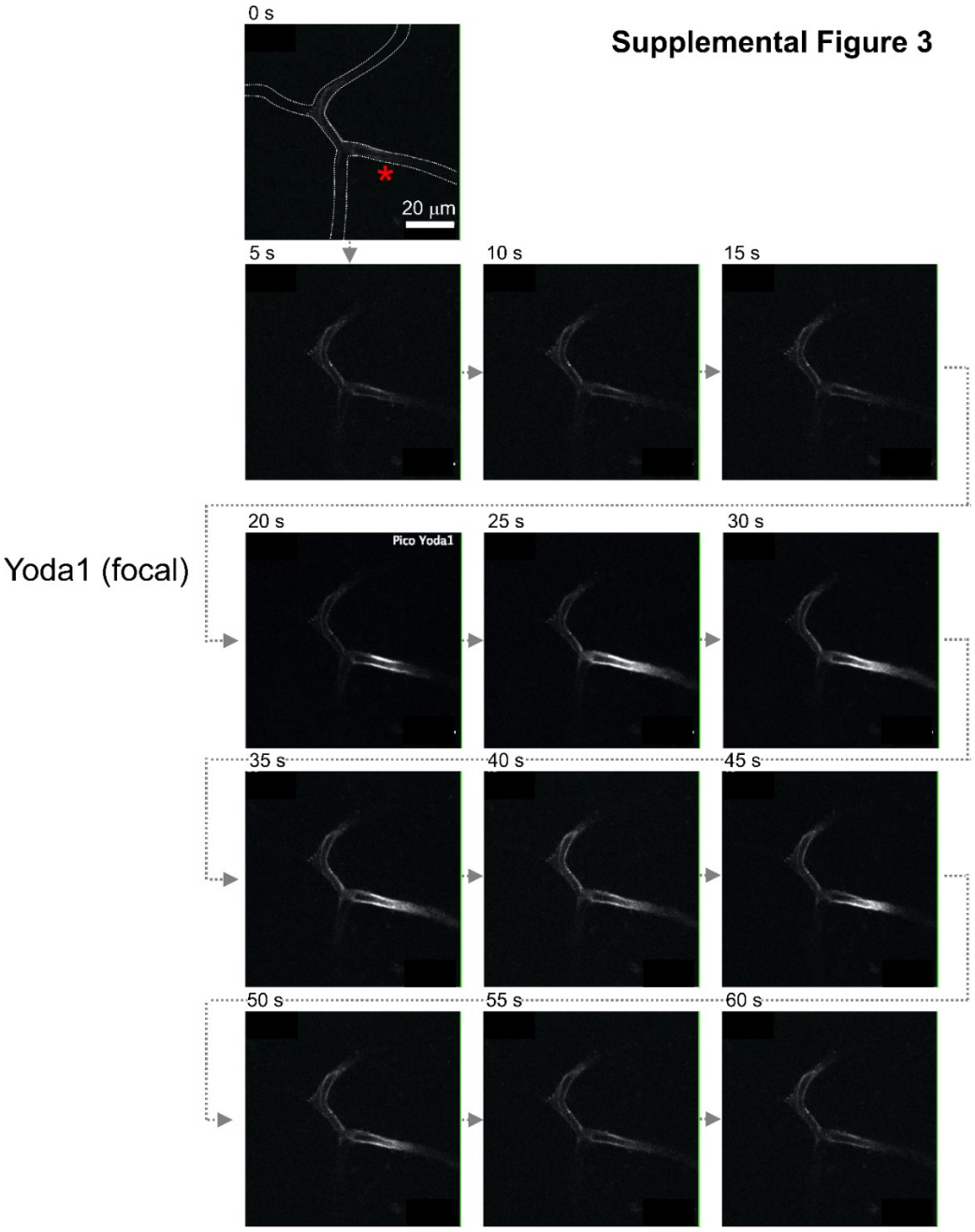

Figure S3. Representative images of Ca^{2+} signals in a capillary segment focally stimulated with Yoda1. Time course of Ca^{2+} fluorescence in a capillary from a pressurized *Cdh5*-GCaMP8 retinal preparation in response to picospritzing 30 µM Yoda1. The location of the tip of the picospritzing pipette is marked with a red "*". Focal application of Yoda1 lasted 3 s at a low ejection pressure (<3 psi).

Supplemental Figure 4

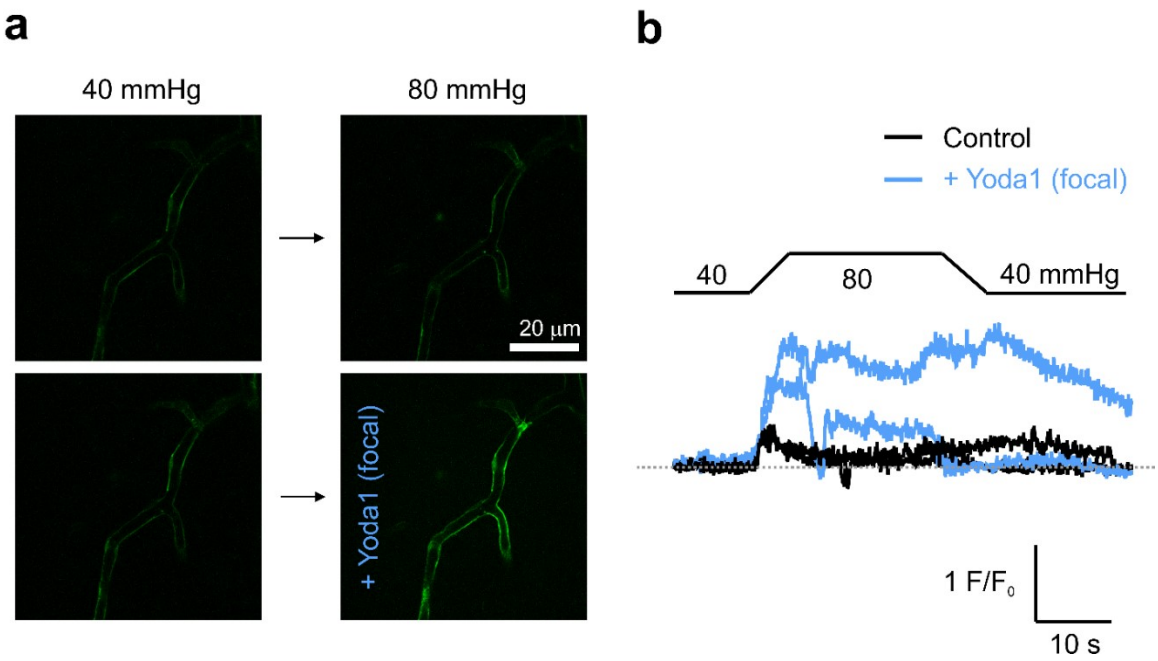

Figure S4. Mechano-pharmacologically induced endothelial Ca^{2+} transients are driven by Piezo1 channels. (**a**) Imaging field of a capillary in a pressurized retina preparation at baseline (40 mmHg; *left*) and after elevating intra-ophthalmic artery pressure from 40 to 80 mmHg (*right*) showing Ca^{2+} responses in the absence (top; control) and presence (bottom) of focally applied Yoda1 (30 µM; 30 s exposure, 3 psi). (**b**) Traces showing Ca^{2+} transients in the target capillary before, during and after the pressure-change sequence (40-to-80-to-40 mmHg) under control conditions (black) or with concurrent focal application of Yoda1 (blue). Conditions are as described in *a*.

SUPPLEMENTAL MOVIES

Movie S1: Whole *Cdh5*-GCaMP8 retina imaging while changing the intravascular pressure at the ophthalmic artery from 20 to 80 to 20 mmHg. *Left*: control condition; *Middle*: perfusion solution was supplemented with 30 µM Yoda1; *Right*: perfusion solution was supplemented with Yoda1 and 10 µM ruthenium red.

Movie S2: Focal stimulation of a capillary segment in a pressurized retina (40 mmHg) with 30 µM Yoda1. *Green*: Ca^{2+} transients; *Red*: Yoda1 solution supplemented with TRITC-dextran; *Blue*: endothelial cell outline.

Movie S3: Focal stimulation of a capillary segment in an unpressurized retina (0 mmHg) with 30 µM Yoda1 (as in movie S2).

Movie S4: A change in pressure at the ophthalmic artery of a retina preparation (40 to 80 mmHg) evoked Ca^{2+} transients in a capillary segment.

Movie S5: Similar to Movie S4 in addition to the simultaneous focal application of Yoda1 (30 µM) onto the capillary of interest.