Supplemental Experimental Procedures

Fly Stocks

The *nompC* mutant lines were provided by C. Zuker (Columbia U.). Class III da neuron Gal4 driver (19-12-Gal4) was isolated from a Gal4 insertion library provided by U. Heberlein (Janelia Farm). The transgenic fly lines carrying wild-type NOMPC and mutated NOMPC proteins (Δ1-29ARs and Δ1-12ARs) are as previously described (Cheng et al., 2010).

Constructs of mutated NOMPC channels

The full length NOMPC, Δ1-29ARs and Δ1-12ARs have been previously described (Cheng et al., 2010), corresponding to NOMPC-L, Δ29ANK and Δ12ANK, respectively.

To generate NOMPC Ankyrin repeats deletion constructs, a PCR based approach was used. For Δ1-12ARs-NOMPC-GFP, aa126-533 (aa: amino acid, reference sequence HM582118) was removed and a linker of two amino acids ArgSer was added to link aa1-125 to aa534-1732, eGFP was fused on the C-terminus before the stop codon. In short, the sequence can be summarized as aa1-125+ArgSer+aa534-1732+eGFP). A similar strategy was used to generate other constructs, and the protein sequence can be summarized as follows:

Δ1-29AR-NOMPC::GFP (aa1-125+ArgSer+aa1144-1732+eGFP); Δ13-29AR-NOMPC::GFP (aa1-533+ArgSer+aa1144-1732+eGFP); Δ16-29AR-NOMPC::GFP (aa1-656+ArgSer+ aa1144-1732+eGFP); Swap AR::GFP (aa1-125+ ArgSer+aa534-1143+LeuILeLys+aa126-533+ArgSer+aa1144-1732+eGFP); 29+29ARs-NOMPC::GFP

(aa1-125+ArgSer+aa126-1143+LeuIleLys+aa126-1143+ArgSer+aa1144-1732+eGFP); 29+17ARs::GFP (aa1-533+ArgSer+aa126-1143+ArgSer+aa1144-1732+eGFP). The mutated NOMPC coding regions were cloned into pUAST vector for cell transfection and transgenic fly injections.

Generation of the synthetic mechanogated potassium channels

The fragment of NOMPC Ankyrin repeats with linker region (nucleotide acid 1-3804) was amplified from full-length NOMPC cDNA via primers: 5'-agtccagtgtggtgggaattcatgtcgcagccgcgcgga-3' and 5'-gcaggcccggatgcccacgtcagggagc-3'. The fragment of Kv1.2 trans-membrane domains (478-1500) was amplified from full-length rat Kv1.2 cDNA via primers 5'-gggcatccgggcctgccaggattatagc-3' and 5'-tgctggatatctgcagccgcgggacatcagttaacattttggtaatattcacatagtttgtg-3'. The fragment of Kv2.1 trans-membrane domains (544-2019) was amplified from full-length rat Kv2.1 cDNA via primers 5'-tccctgacgtgggcatccgtggccgccaagatcctg-3' and 5'-aattctaga gatactctgatccctagtgctccc-3'. Fragments of NOMPC Ankyrin repeats with linker and Kv1.2 or Kv2.1 trans-membrane domains were assembled into pAc5.1/V5-His A (Invitrogen) with C-terminus GFP by following the protocol of Gibson Assembly Kit (NEB). The fragment of NOMPC Ankyrin repeats without linker region (1-3423) was amplified from full-length NOMPC cDNA with primers: 5'-agtccagtgtggtggaattcatgtcgcagccgcgcgga-3' and 5'-gcaggcccctccatcaggccgtaggtgtcg-3'. The fragment of Kv1.2 trans-membrane domain and C terminal tail (478-1500) was amplified from full-length rat Kv1.2 cDNA. 5'-tgatggaggggcctgccaggattatagc-3' and 5'-tgctggatatctgcagccgcgggacatcagttaacattttggtaatattcacatagtttgtg-3'. The fragment of

Kv2.1 trans-membrane domain and C terminal tail (544-2019) was amplified from full-length rat Kv2.1 cDNA. 5'-gacacctacggcctgatggtggccgccaagatcctg-3' and 5'-aattctaga gatactctgatccctagtgctccc-3'. Fragments of NOMPC Ankyrin repeats without linker and Kv1.2/Kv2.1 trans-membrane domains were assembled into pAc5.1/V5-His A (Invitrogen) with C-terminus GFP by following the protocol of Gibson Assembly Kit (NEB).

S2 cell staining

For non-permeabilized staining, the antibody (rabbit anti-NOMPC-EC (1:1000; generated against an extracellular epitope, amino acid residues 1469-1488; YenZym Antibodies, LLC)) was diluted in Schneider's *Drosophila* Medium and incubated with transfected cells for 30 min at 25 ℃. Cells were then washed 3 times with PBS and fixed with 4% PFA for 30 min at 4 ℃. Cells were blocked with 10% Normal Goat Serum for 30 min at room temperature and then incubated with secondary antibody (Alexa 555 conjugated anti-mouse IgG 1:200, Invitrogen) for 30 min. After washed briefly, cells were mounted in coverslip for imaging.

For permeabilized staining, cells were fixed and incubated with PBST (PBS+0.1%Triton) for 10 min. Then the cells were blocked and stained with primary and secondary antibodies.

For all microtubule staining, the cells were fixed with 4% PFA for 20 min at RT and incubated with tubulin antibody (DM1a, 1:1000) for 30 min at 25℃ sequentially after NOMPC antibody incubation (non-permeabilized staining) or simultaneously with NOMPC antibody (permeabilized staining).

For nocodazole treatment, cells were pre-treated in PBS with 100 nM nocodazole for 30 min before proceeding to non-permeablized staining as described above.

Images were acquired with Leica SP5 confocal microscope or Zeiss Pascal 510 microscope. Surface expression levels of NOMPC channels were measured by quantifying the fluorescence intensity of single cell area with ImageJ software.

TIRF microscopy

TIRF microscopy was carried out as previously described (McGorty et al., 2013). Briefly, the system is custom-built from a Nikon Eclipse Ti-E inverted microscope. Lasers (488 nm, Vortran Lasers; and 561 nm, Sapphire 561–200, Coherent), a dichroic mirror (zt405/488/561/640rpc, Chroma or T660lpxr, Chroma) and an appropriate band-pass filter (ET525/50 m, ET595/50 m, ET702/75 m, Chroma) are used for multi-color imaging. Images are recorded with an electron multiplying CCD camera (EMCCD, iXon+ DU897E-C20-BV, Andor) using a custom-written software in Python. Images were processed with ImageJ software.

Larval neuron staining

Larval body wall neuron immunohistochemical staining was performed as reported previously (Grueber et al., 2002), except that the mounting medium used was VectaShield (Vector Laboratories). Briefly, the tissue was kept non-permeabilized with the exclusion of detergent throughout the immunostaining procedure. The filleted larvae was fixed on ice for 20 min and blocked with blocking buffer for 30 min RT. The samples were then incubated in PBS containing NOMPC antibody (α-NOMPC-EC, 1:500) for 2 hr RT and then with secondary antibody for 30 min RT. Slides were imaged on a Zeiss Pascal 510 confocal microscope using an oil immersion 40X objective. The surface NOMPC level was quantified with ImageJ by

measuring the fluorescence signal intensity over major dendrite branches of class III neurons.

Expression and purification of NOMPC full-length protein

Drosophila nompC gene was subcloned into a modified BacMam vector with a mammalian cell active promoter (Liao et al, 2013) for baculovirus transduction-based expression in HEK293S GnTi- cells. A Strep tag II was added to the C-terminus for affinity purification. Recombinant baculoviruse was produced following the manufacturer's protocol (Bac-to-Bac expression system, Invitrogen). NOMPC was expressed in HEK 293S GnTi- cells by transducing the cells with baculovirus harboring the gene encoding NOMPC with a C-terminal GFP and Strep tag II under the control of cytomegalovirus promoter. Protein expression was boosted 24 hours post transduction by adding sodium butyrate to a final concentration of 10 mM. Cells harvested 48 hours post transduction were homogenized in a buffer composed of 300 mM KCl, 50 mM Bicine, 2% LDAO (Anatrance), 10% glycerol, 1 mM PMSF and protease inhibitor cocktail (Sigma) at pH 8.5. The lysate was then agitated for 2 hours prior to removal of insoluble fractions by centrifugation at 15,000 g for 30 minutes. The supernatant was incubated with Streptactin resin (IBA) for 4 hours. Afterward the resin was washed with 20 column volumes of the wash buffer (300 mM KCl, 50 mM Bicine, 0.2% LDAO, pH 8.5). NOMPC was eluted with the wash buffer containing 5 mM d-Desthiobiotin (Sigma). Protein quality was examined with Coomassie staining.

Co-sedimentation

Tubulin (Cytoskeleton, HTS02-B) at 2.2 mg/ml in G-PEM buffer (1 mM GTP, 1X BRB80 buffer: 80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA) was polymerized by

addition of 1 mM DTT and 20 μM paclitaxel at 37 ℃ for 2-3 hr without shaking. Polymerized tubulin was centrifuged at 80 K in the TLA100 rotor for 10 min at 25 ℃ and resuspended in 80 μl of resuspension buffer (1X BRB80 buffer, 1 mM DTT, 20 μM paclitaxel). Protein concentration was measured with Bradford assay. Tubulin at 1mg/ml in resuspension buffer was incubated 30 min to 1 hr at room temperature to repolymerize.

During this incubation cell lysate or purified protein of interest were spin down at 80K for 10 min to ensure that all non-specific protein aggregation was removed. Supernatant was quantified with Bradford assay. Equal amounts of protein were added to the polymerized microtubules or resuspension buffer alone as negative control. Addition of 4X co-sedimentation buffer (4X BRB80, 4 mM DTT, 20 μM paclitaxel) to the incubation mix ensured maintaining microtubule polymerizing conditions after addition of protein samples. The mix was incubated at room temperature for 20 min and spin at 60 K for 10 min. Supernatant and pellet resuspended in equal volume of the resuspension buffer were collected and analysed by western blot, comparing the distribution of the protein of interest between pellet and supernatant fraction in conditions with or without microtubules.

Immuno-electron microscopy

Halteres and attached fragments of the thorax were fixed at 4 ℃ in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer at pH 7.3 for 4 hrs. Halteres were then washed in PBS and postfixed with 2% aqueous OsO_4 at 4 ℃ overnight. The samples were then dehydrated in an ethanol series including a block staining step with uranyl acetate in 70% ethanol for 1 hr. Infiltration with Fluka Durcupan was done for two days raising the Durcupan concentration from 30% to

90% the first day and three exchanges of pure Durcupan the second day. Before thin embedding between two glass slides the remaining thorax fragments were removed from the halteres.

70 nm ultrathin sections were cut with a Reichert Ultracut E ultramicrotome and transferred onto Formvar-coated copper mesh grids (PLANO G2405C). Poststaining was done for 40 min with 4% (w/v) uranyl acetate in water and for 2 min with lead citrate (Reynolds, 1963). Micrographs were taken with a JEOL electron microscope (JEM 1011, JEOL, Eching, Germany) with a GatanOrius 1200A camera (Gatan, Munich, Germany).

S2 cells recordings

Drosophila S2 cells were cultured in Schneider' *Drosophila* medium supplied with 5% FBS at 25 ℃. Effectene Kit (Qiagen) was used to transfect cells according to the product protocol. pUAST-NOMPC-GFP (wild-type or mutants) constructs were co-transfected with pActin-Gal4. Cells were plated onto ConA-coated coverslips in 35 mm petri dishes before recording. Recordings were carried out 1-2 days after transfection.

Transfected cells were identified by fluorescence. Patch electrodes with 5-7 MΩ resistance were used. Cell-attached mode was obtained by applying negative pressure to the pipette when they reached the cell surface. Three recording modes were achieved by different manipulations. Inside-out mode was formed by rapidly pulling the pipette away from the cells. Whole-cell mode was achieved by rupturing the cell membrane with a brief suction; Outside-out patch mode was obtained after the forming of whole-cell mode by withdrawing the pipette slowly from the cells. Sample

rate was 10 kHz and filtered at 2 kHz (low-pass). The pipette solution contains: 140 mM potassium gluconic acid (140 mM CsMES (cesium methanesulfonate) for Cs^+ solution), 10 mM HEPES. The bath solution contains: 140 mM NaMES (sodium methanesulfonate), 10 mM HEPES. All solutions were adjusted to 320 mOsm and pH 7.2. The single channel conductance was then calculated as: $I / (V_m-E_{rev})$, where V_m is the holding potential, I is the single channel current amplitude and E_{rev} is the reversal potential of the current.

All amplitudes were measure at -60 mV and E_{rev} was calculated as previously described (Yan et al., 2013). The I-V curves were plotted and fitted from the current amplitude measured from -100 to 100 mV with 20 mV increments.

Drug application

Nocodazole was dissolved in the bath solution to the final concentration right before experiments. The drug-containing solution was perfused to the recording chamber. A repetitive negative pressure was applied to the inside-out patch every 5 s before and after nocodazole application. Similar experiments were carried out with other drugs. The concentration used are: nocodazole (Cayman), 100 nM; colcemid (Cayman), 10 μM; paclitaxel (Cayman), 10 nM; cytochalasin D (Sigma) 10 nM, latrunculin A (Cayman) 1 μM, jasplakinolide (Cayman), 100 nM; MTX (maurotoxin, Sigma, 1 nM).

Larval electrophysiological recordings

Fillet preparations were made by dissecting 3rd instar larvae in haemolymph-like saline containing (in mM): 103 NaCl, 3 KCl, 5 TES, 10 trehalose, 10 glucose, 7

sucrose, 26 $NaHCO_3$, 1 NaH_2PO_4 and 4 $MgCl_2$, adjusted to pH 7.25 and 310 mOsm. 2 mM Ca^{2+} was added to the saline before use. Muscles covering the neurons were gently removed with a fine forceps and the body wall neurons were exposed. Class III da neurons were visualized and identified by fluorescent markers (or by GFP-tagged NOMPC channels) driven by the 19-12-Gal4 driver. Glass electrodes for electrophysiological recording were pulled with a P-97 puller (Sutter instruments), and filled with external saline solution (as above). The action potentials were recorded extracellularly with a sample rate of 10 kHz and low-pass filtered at 2 kHz. Multiclamp 200B amplifier, DIGIDITA 1440A and Clampex 10.3 software (Molecular Devices) were used to acquire and process the data. The extracellularly recorded action potentials were detected by threshold-based search on the single-unit recordings. A fixed-length time window (1 second) was taken before and after stimulation onset time. The response firing number was calculated by the difference of the two time windows.

Mechanical stimulation with piezo actuator

A glass probe was driven by a piezo actuator mounted on a micromanipulator to deliver mechanical stimulation. The movements were triggered and controlled by the piezo amplifier, which was synchronized with the programmed signals from pClamp software (Molecular Device). For larval body wall stimulation, the stimulation pipette was sealed and fire-polished to a diameter around 20 μm. The stimulation pipette was driven downward vertically to press the larval body wall to specified displacements for 1 second.

For cultured S2 cells, the pipette was sealed and polished by microforge to a diameter around 1 μm. The probe was controlled by piezo attenuator and moved along an angle

of 45 degrees to the cell surface. To apply different forces, a series of mechanical steps in 0.4 μm increment were applied every 8 s. Cells were held at -60 mV for all stimulations.

High speed pressure clamp

Negative pressure was applied to the membrane patches via a High Speed Pressure Clamp (HSPC, ALA-scientific). Signals generated from pClamp 10.3 software were sent to HSPC to control the timing and intensity of the pressure.

To record the dose-response curve of the mechanosensitive current, pressure steps with 10 mmHg increment were applied to the membrane patch through the recording pipette.

To test the time course of nocodazole effects, a brief pressure pulse was applied to the membrane every 5 s before and after drug application.

Behavior assay

Touch assay was carried out as previously described (Yan et al., 2013). Briefly, 96 hours after egg laying, third instar larvae were gently picked and allowed to crawl freely at room temperature. The larvae were touched with an eyelash at one side of the thoracic segments gently and the behavioral responses were scored. Larvae that did not respond to touch were scored 0, those larvae that stopped or hesitated were scored as 1, those that retracted their heads or turned were scored as 2, those that retracted with a single wave were scored as 3, and those that retracted and moved in the opposite direction were scored 4. Each larva was touched and scored four times and

the values were summed as the final score.

Reference

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