

Supplementary Information for

Nanchung and inactive define pore properties of the native auditory transduction channel in *Drosophila*

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Fig. S1. Lch1 neurons respond to mechanical stimuli with action potential firings. (*A*) Touching the dendrite tip of a lch1 neuron with a 1 μ m displacement induced a burst of action potentials. (*B*) Summary of action potential firings of lch1 neurons in response to progressively greater mechanical stimuli. Δ Spike Frequency: increase in the frequency of action potentials after stimulus onset compared to 100 ms before stimulus onset (n = 6, 11, 11, 8, 8, 6, 7). The displacements were from 0.2 μ m to 1.4 μ m with 0.2 μ m increments. Error bars, mean ± SEM.



Fig. S2. No mechanical stimuli-induced currents are detected in lch1 neurons of *nan* and *iav* mutants in response to increasing stimuli. Lch1 neurons were held at -60 mV. *nan*^{36a}, *Nanchung* null mutant; *iav*¹, *Inactive* null mutant. 1 µm, 2 µm, 3 µm, 4 µm, and 5 µm represent the displacements of mechanical stimuli. Genotypes are as follows: for *iav*¹: *iav*¹/y; lav-Gal4/+; UAS-CD8-GFP/+. For *nan*^{36a}: lav-Gal4/UAS-GFP; *nan*^{36a}/ *nan*^{36a}.

Fig. S3. Sound-induced currents in Cho neurons. Representative traces of current responses to a 500Hz 90 dB pure tone sound stimulation in wild-type and nan^{36a} , iav^1 mutants. Lch1 neurons were held at -60 mV. The sound-elicited transduction current is similar to those recorded from locust auditory receptor neurons in a previous study (1). The grey line overlying the trace represents a single exponential fit of adaptation of the sound-induced current.

Fig. S4. Representative traces of single channel activities in control, NAN and IAV transfected S2 cells. S2 cells were clamped at potentials ranging from -100 to +100 mV (holding potentials) with 20 mV increments and recorded with a K⁺-based internal solution and a Na⁺-based extracellular solution. Ctrl, nontransfected S2 cells; NAN, S2 cells transfected with NAN-Flag; IAV, S2 cells transfected with IAV-mCherry.

Fig. S5. Representative traces of single channel activities in Inactive-mCherry- and Nanchung-Flag-coexpressing S2 cells with or without NAM. The concentrations of nicotinamide (NAM) in the K⁺-based pipette solution during the whole-cell recordings were 0, 0.25 μ M, 0. 5 μ M, 1 μ M, 2 μ M, and 4 μ M. The holding potential was -60 mV.

Fig. S6. Representative traces of single channel activities in empty, NAN and IAV transfected S2 cells with NAM. S2 cells were clamped at potentials ranging from -100 to +100 mV (holding potential). Ctrl+NAM, nontransfected S2 cells; NAN+NAM, S2 cells transfected with NAN-Flag; IAV+NAM, S2 cells transfected with IAV-mCherry. All the K⁺-based pipette solutions contained 2 μ M NAM.

Fig. S7. NAN-IAV single channel activities with voltage steps in S2 cells. (*A*) Single channel current traces of NAN-IAV with voltage steps in the extracellular Na⁺/ intracellular K⁺ condition. NAN-IAV, spontaneous single channel activity in normal Na⁺/K⁺ solution; NAN-IAV+NAM, active single channel current with 2 μ M NAM in internal solution. Cells were clamped at potentials ranging from -100 to +100 mV (holding potential). (*B*) I-V curves of NAN-IAV single channel currents in Na⁺/K⁺ conditions with 2 μ M NAM in pipette solution (n = 5). Error bars, mean ± SEM. The liquid junction potential was +4.5 mV; the holding potentials were from -100 mV to +100 mV; the membrane potentials (X axis) were from -104.5 mV to +95.5 mV with 20 mV increments.

Fig. S8. Single channel current traces of NAN-IAV with voltage steps in bi-ionic conditions in S2 cells. S2 cells were clamped at potentials ranging from -100 to +100 mV (holding potentials). Na⁺/Cs⁺, extracellular Na⁺/intracellular Cs⁺; K⁺/Cs⁺, extracellular K⁺/intracellular Cs⁺; Ca²⁺/Cs⁺, extracellular Ca²⁺/intracellular Cs⁺. All the internal solutions contained 2 μ M NAM.

Fig. S9. Electrophysiological recordings of NAN-IAV or NOMPC in S2 cells. (*A*) Representative traces of control, NAN-IAV or NOMPC transfected S2 cells for whole-cell patch recordings in response to 2-8 μ m mechanical displacements. The black arrow denotes the direction of mechanical displacements. The holding potential is -60 mV. (*B*) Representative traces of control, NAN-IAV or NOMPC transfected S2 cells for outside-out patch recordings in response to -20– -120 mmHg negative pressure. The black arrow denotes negative pressure. The holding potential is -60 mV. (*C*) Representative traces of control, NAN-IAV or NOMPC transfected S2 cells for outside-out pressure. The holding potential is -60 mV. (*C*) Representative traces of control, NAN-IAV or NOMPC transfected S2 cells for outside-out pressure. The holding potential is -60 mV. (*C*) Representative traces of control, NAN-IAV or NOMPC transfected S2 cells for outside-out pressure. The holding potential is -60 mV. (*C*) Representative traces of control, NAN-IAV or NOMPC transfected S2 cells for outside-out patch recordings in response to +20– +120 mmHg positive pressure. The black arrow denotes negative pressure.

Fig. S10. Point mutations of NAN and IAV in selective filter region altered the reversal potential of NAN-IAV channels in S2 cells. (*A*) Representative traces of single channel activities in wild-type and mutant NAN-IAV channels at -60 mV (holding potential) in Na⁺/Cs⁺-based solutions with 2 μ M nicotinamide (NAM) in internal solution. Gray dashed lines denote the closed states of single channel. The liquid junction potential is +4.9 mV; the holding potentials are -60 mV, 0 mV, +60 mV; the membrane potentials are -64.9 mV, -4.9 mV, +55.1 mV. (*B*) Average current-voltage relationship of wild-type and mutant NAN-IAV single channel currents in Na⁺/Cs⁺-based solutions with NAM (n = 8, 7, 7; mean ± SEM). The liquid junction potential is +4.9 mV; the holding potentials are from -100 mV to +100 mV; the membrane potentials (X axis) are from -104.9 mV to +95.1 mV with 20 mV increments. NAND681A-IAV, S2 cells transfected with NAN-D681A-Flag and IAV-mCherry; NAN-IAV-D615A, S2 cells transfected with NAN-Flag and IAV-D615A-mCherry. (*C*) Summary of reversal potentials of single channel currents of wild-type and point mutant NAN-IAV in S2 cells (n = 8, 7, 7; mean ± SEM). One-way analysis of variance followed by Holm–Sidak post hoc analysis was used for comparison among multiple groups. ***p < 0.001.

Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	1 1 1 1	MKTELKKCERKKA PEMKPGÄ I LDAV I SOKQAGVSTQALYKFVNL MGG LLVDMMKRACQTKQFAE I DHA I KTKVEPFL NK MKFLLKKCERKKA PEMKPGÄ I LDAV I SOSSATACKCLLYKLADY RG DLI DA I NSGG L I AVEQL I REOFGYFMY ND MKMKKGSTD I DETETCASVET DESHSDDTNRSTQENRKLKFCQAKYSI FSSP MKGRAFGKGATETN I APMADSYQ I MKMKKGSTD I DETETCASVET DESHSDDTNRSTQENRKLKFCQAKYSI FSSP MKGRAFGKGATETN I APMADSYQ I MKMKKGSTD I DETET.CASVET DESHSDTNRSTQENRKLKFCQAKYSI FSSP MKGRAFGKGATETN I APMADSYQ I MKGSTD I DETET.CASVET DESHSDTNRSTQENRKLKFCQAKYSI FSSP MKGRAFGKGASEGKGASEGKGASE	66 77 81 42 67
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	67 78 82 43 68	GAGRYFFISKLVLLRNRDRPRTRQ	134 127 179 127 165
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	135 128 180 128 166	RGAVGET LIHLCLLNASSLHADLAKRILKFYPKLILDITMSDETY GESVLHIATVNEDPANVKYTLDANADVOERCCGARMSAEDTKFSRTDSPDHEYVA RGALGESLLHVII COSKVHTKLARVLRVPNLALDVMEGEEVLGASLHLSIAYSNNE VADITAGADTHORIGSFLPRDDORA	234 217 258 205 244
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	235 218 259 206 245	LCPMTNYDGYVYVGEYPL FAAGLS EECFRLVIARG ADPDF0DTNON IVLEMLVIYEKI ENFDV BYEYGTNIHIKNION PAKSTDYEGLAMGEYPL AWAA CGANESYYNLIYDGG SDPDADDS SON HILLMYVYORKL	315 301 348 295 334
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	316 302 349 296 335	TPLITA KLORVEM FHVMSIEREIYWQLESITCAAYPILM DTINEETGNINKDSVINFVYFG. DKLEHLELDGVVI.DLIKTWD TPLITACKLORAEVREMEELSAR	401 387 440 385 427
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	402 388 441 386 428	TECKSREYKOEYMFALTE ISLESEIL PGPDAKDEDEDGANSTTAKSDLYRON GSDSYHLHSKRATMTTEYKTFWLNFTEYYDPSEVEVLPAW TEMONOGLKRLIILSTHLICISVSVYL PAHDGEAEDEDSEGSDASAAALDIQSDEGGGOYNAG	495 455 480 429 467
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	496 456 481 430 468	WESYAQCPLINLESDLÄKLE IMAE LINFVGA I LYLLVA-LREA-FFLG KMFIEN MT PSRVKELFSCAL MMT I PWLRVSCLTE I DDHVTVV I MLTTAP TVARY AFFA I LVGVLSVVI FOQGDE I-KNOG SÄFLKOLSM-PAKAI ELFSNLTI ACIPFRI ISDT-DTEEN I I AVPGS NSVTRT GELI TVIGT VFFRSI BYFTORSPSKA-L LADSVYFFLGATSIFLL ISTI VFCGR-NEVVFFU TICLMS SREGYLLI GHIISITG FYFFRSI DYFORPSKS-L AVPGSIFFASVVI VCYGO-YFVLFFU TICLMS TVGDYFN GELSVSGVVFFRGI DYFLOHRPSKS-LFVDSYSEL EVDSIFM VSVV VCYGO-YFVLFFU LCLALS S2 S3 S4	593 536 559 510 548
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	594 537 560 511 549	- YFLFFCRGFKTVGPFVTMTYRNVMGDLLREVS V VFVMGE OG YIIFLTFDNPSSPEDQDA- WFLLMFFAGAIRLTPFVTMTYSN TGONFTGIIVCIVLGGS OG YFLFFTVK6HPOVOST- WANVIYTRFGFOLMGTSSMTEKLLSSMVFRHFVULFFGGAALVTTLGGGGRTDL- NSTC- WINLLYFSRGSNLGTNNMTVGETRFELVYVVFTGGSAALVTTLDQESIDSOSTDFRLSEDIPSLNPTPDSSNPOSRMTHNQTTARDGRGR WTNMLYYTRGFQOMGTAALMIEKNILRDCREMFVYVFFGGSTVVTT 100051D505TDPRLSEDIPSLNPTPDSSNPOSRMTHNQTTARDGRGR WTNMLYYTRGFQOMGTAALMIEKNILRDCREMFVYVFFGGSTVVTT	656 596 623 610 616
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	657 597 624 611 617	ESNPMPSPMESIVAMELMSLTNFGTYV-GAMVSTQHEYEANILFFLFNVIVSVL VNMLTAMIGNTYOKTA - IRNENOPOW MENTITT - STMALEQ	736 672 709 701 701
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	737 673 710 702 702	R IVL VV RSVPPAERLKNFMOYSQSMSDG ···· RR ···· ALVLRL · NMTEEEK E ···· MKEVQEMKR ··· I HQRFAKK KI VV TL RAVEQADAKGVLEAVSI PT BPS ··· DDSGFEVRG MVI KS · KSKTRAKOR ··· KGAVSMWRRVGRVTLTALKK I TL DI SETLMSFRDTFRSKSVLOG FTPDGKDVRVGFRVDE NWKKWNSNIG I · I KDPONCHOLKSTLSASFRP ··· RGKRWSL ··· VPK ··· K I TT DM WI LEKCLOGKLRSGEEKDLOG ·· GQEPDRWCFSVEE NWTOWNRNNG I I IN DPGKCTODPSPANVOREPSRGVLOT ··· FSRRRT ··· Q I TT DT KSELKSKLOVG FTPDGKDDYWCFRVDE NWTWNTNYG I · IN DPGNCGVKRLSFSLRSGRVSGRWKNFALVPLL ··· R	800 746 800 793 797
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	801 747 801 794 798	ROMEREARALRRQQEYEKFFGTAPKSECSDNNNF. RGMIGEEMRRLMNGRASISSPVKVTKOKLKDPYNLHTDSDFTNAMDMLTFASNPASSNGVTLRSVTAPPPAPPAPDPFRELIMMSDQRPET ETNVKIDNETVPEEIPLQQKPTLADQTVPEEDQEVTSKAE RAOTREGHELSPLAEASSSVPTLADQTVPEEDQEVTSKAE DASTRDRHATQQEEVQLKHYTGSLKPEDAEVFKDSMVPGEK	834 837 840 813 838
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	838	HDPHYFAGLQQLANKAFDLVEQTMKTQPQAPVAKKVDPLPVASVAKASPAAPATQATATAAAASDLMAMPLPISNLSNLFQDPKDIVDPKKLEEFMAMLA	937
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	938	V EVETEESDSGGPILGKLSLAKRTHNALSKAEIRRDQQGFEGHSHGQFQPMSSVWAPPGLDVDTGFHFDEAVAEEVLTIEQEAEVETEDGNGGQDSEDIPT	1037
Drosophila NAN Drosophila IAV X. tropicalis D. rerio R. norvegicus	1038	AEEVHATMKQFHLRKCQPAQDEAARRAKSARVRRNKVSPEQSDDPDERSQRGRSAYTRRTQSPPDPLEPWSTRELQDINKILARK	1123

Fig. S11. Sequence alignment of TRPV Orthologues. The sequence homology of *Drosophila* IAV and NAN to other TRPV1 orthologs was analyzed by Clustal Omega. Secondary structure elements and the C-terminal region of IAV are indicated below the sequence. Red arrowheads mark the two residues 857 and 990.

	ΔAR1-NAN-IAV	ΔAR2-NAN-IAV	ΔAR3-NAN-IAV
100 mV		ۅڛٵڣ ٦٩٩٩ من _{الم}ين ٢٩٩٩ من الم ين ٢٩٩٩ من ٢	
80 mV			
60 mV		• • • • • • • • • • • • • • • • • • •	
40 mV			
20 mV			
0 mV		,	
-20 mV			
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-60 mV		······	
-80 mV			
-100 mV		· · · · · · · · · · · · · · · · · · ·	
	ΔAR4-NAN-IAV	ΔAR5-NAN-IAV	ΔAR6-NAN-IAV
100 mV			
80 mV			
60 mV			
40 mV			
20 mV			
0 mV			
-20 mV			·····
-40 mV		·	
-60 mV			·····
-80 mV			
-100 mV			
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			200 113

Fig. S12. Representative traces of single channel activities in truncated NAN transfected S2 cells. S2 cells were recorded at holding potentials ranging from -100 to +100 mV with 20 mV increments in Na⁺/K⁺-based solutions. Δ AR, deletion of ankyrin repeats; Δ AR1-NAN-IAV, S2 cells transfected with Δ AR1-NAN-Flag and IAV-mCherry; Δ AR2-NAN-IAV, S2 cells transfected with Δ AR2-NAN-Flag and IAV-mCherry; Δ AR3-NAN-IAV, S2 cells transfected with Δ AR3-NAN-Flag and IAV-mCherry; Δ AR4-NAN-Flag and IAV-mCherry; Δ AR5-NAN-IAV, S2 cells transfected with Δ AR4-NAN-Flag and IAV-mCherry; Δ AR5-NAN-IAV, S2 cells transfected with Δ AR6-NAN-IAV, S2 cells transfected with Δ AR6-Cherry. All the pipette solutions contained 2 μ M NAM.

	NAN-ΔAR1-IAV	NAN-ΔAR2-IAV	NAN-ΔAR3-IAV
100 mV			
80 mV			فيالجميه البراي الباعدي ويتريه والالان والمعالية البادية والانتقال
60 mV			
40 mV			
20 mV			والمرجع والمرجعة الأوري والمترار فأعتاكم ومرجع والمرجع والمتراجعة
0 mV			
-20 mV			
-40 mV			
-60 mV			
-80 mV			
-100 mV			
	NAN-ΔAR4-IAV	NAN-ΔAR5-IAV	NAN-ΔAR6-IAV
100 mV	^ى ئەركىيە ئارىكە ئىيەتلىرىن <u>ئەركىيە تەركە ئىيەت بىرىكە ئاركە ئەكەر بەركىيە</u>		والمحمور والمرجوعة والمستجد ومحمود المتراخية الجروا الجروب المتراجع والمحمود المرجوع والمحمود المرجوع والمحمو
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40 mV			
20 mV			
0 mV			
-20 mV			
-40 mV			
-60 mV			
-80 mV			
-100 mV			
			4
			200 ms

Fig. S13. Representative traces of single channel activities in truncated IAV transfected S2 cells. S2 cells were clamped at holding potentials ranging from -100 to +100 mV. Δ AR, deletion of Ankyrin repeats; NAN- Δ AR1-IAV, S2 cells transfected with NAN-Flag and Δ AR1-IAV-mCherry; NAN- Δ AR2-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR2-mCherry; NAN- Δ AR3-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR3-mCherry; NAN- Δ AR3-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR3-mCherry; NAN- Δ AR4-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR3-mCherry; NAN- Δ AR5-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR5-mCherry; NAN- Δ AR6-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR5-mCherry; NAN- Δ AR6-IAV, S2 cells transfected with NAN-Flag and IAV- Δ AR6-mCherry. All of the K⁺-based pipette solutions contained 2 μ M NAM.

Fig. S14. Immunostaining of S2 cells expressing NAN-IAV and NAN- Δ 857-1123-IAV. Representative images of myc staining in NAN-IAV-mCherry- and NAN- Δ 857-1123-IAV-mCherry-transfected S2 cells. Myc tags were inserted behind the Q455 residue of IAV. Scale bar, 10 μ m.

Fig. S15. No MET currents were detected in lch1 neurons of two truncated mutants of *iav* in response to increasing stimuli. (*A*, *B*) Δ 857-1123-iav and Δ 991-1123-iav could not rescue the MET currents of *iav*¹. Lch1 neurons were clamped at -60 mV (holding potential) in Na⁺/K⁺-based solutions. Values of 1 µm, 2 µm, 3 µm, 4 µm, and 5 µm represent the displacements of mechanical stimuli. Genotypes are as follows: for *A*, Δ 857-1123-iav: *iav*¹/y; lav-Gal4/+; UAS- Δ 857-1123-iav-GFP/+. For *B*, Δ 991-1123-iav: *iav*¹/y; lav-Gal4/+; UAS- Δ 991-1123-iav-GFP/+.

Fig. S16. Localization of wild-type and mutant forms of IAV in Ich5 neurons of *iav*¹ mutant larvae. Expression of GFP-tagged constructs were driven by Iav-Gal4 driver. Scale bar, 20 µm. Genotypes are as follows: iav-GFP: *iav*¹/y; Iav-Gal4/+; UAS-iav-GFP/+. Δ 991-1036-iav-GFP: *iav*¹/y; Iav-Gal4/+; UAS- Δ 991-1036-iav-GFP: *iav*¹/y; Iav-Gal4/+; UAS- Δ 991-1036-iav-GFP/+. Δ 1037-1081-iav-GFP: *iav*¹/y; Iav-Gal4/+; UAS- Δ 1037-1081-iav-GFP/+. Δ 1082-1123-iav-GFP: *iav*¹/y; Iav-Gal4/+; UAS- Δ 1082-1123-iav-GFP/+.

Fig. S17. A schematic of auditory transduction in *Drosophila* larval Cho neurons. At the tip of the dendrite, each Cho neuron bears a cilium (Left), which displays an axoneme containing microtubular doublets and dynein arm-like protrusions (Right). Nanchung and Inactive are localized to the proximal cilia, while NompC has a restricted distribution in the distal cilia (Right).

Fig. S18. The scolopidia structure of *Drosophila* auditory organs. The illustration of chordotonal organs was adapted from a previous study (2). Adapted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Neuroscience Bulletin; Axonemal Dynein DNAH5 is Required for Sound Sensation in Drosophila Larvae. Li B, Li S, Yan Z, © 2021. The cilia of Cho neurons, where auditory transduction take place, are surrounded with scolopale space, and the ciliary tips project into cap cells, which are attached to the cuticles through tendon cells.

SI References

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