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LETTER TO THE EDITOR

Molecular Basis for Cephalic Mechanosensitivity of *Drosophila* Larvae

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Dear Editor,

For an animal to explore its environment, the mechanosensors on its head are of particular importance. For example, in *Drosophila* larvae that constantly explore the surrounding substrate during foraging and wandering, the cephalic segments host highly specialized organs with extraordinary mechanical sensitivity [1]. While mechanosensation on the thoracic and abdominal segments has been studied extensively [2], the functions and molecular features of cephalic mechanosensation have not yet been characterized, despite the unambiguous presence of mechanosensors [3].

In *Drosophila*, there are mainly two types of mechanosensory neuron (Types I and II) in the peripheral nervous system [4]. Type I neurons include external sensory (es) organ neurons and internal chordotonal neurons [5]. In contrast, Type II neurons are characterized by elaborate arborizations and are involved in mechanosensation and proprioception [2]. Moreover, the functions of several genes in the transient receptor potential (TRP) family have been demonstrated in larval abdominal gentle touch, sound, and locomotion [2, 6]. It is still an open question whether these genes function in cephalic mechanosensation [7]. Considering the anatomical differences between larval cephalic, thoracic, and abdominal segmental sensory organs, it is critical to determine the expression and function of these genes in larval cephalic sensory organs as well as their functions in mechanotransduction.

Fly larvae show stereotyped responses to gentle touch and the behavior pattern depends on the touch location. Previous studies have used a human eyelash to stroke the thoracic segments from posterior to anterior and quantified the touch sensitivity by scoring each response [8]. Given that such stimulation might be too strong for the larval head, we fabricated a U-shaped polypropylene probe to deliver reliable and mild touch stimuli (Fig. 1A). We found that the score in response to head touch was ~ 10 (summation of 5 trials) in wild-type larvae (Fig. 1B). As a first step to investigate the molecular mechanism underlying the head-touch response, we tested the head touch sensitivity of the mutants from a pool of mechanotransduction channel genes with the above assay (Fig. 1B). Among these genes, *nompC* and nanchung (*nan*) mutant larvae showed a significant defect in the head-touch response (Fig. 1B), while piezo and pickpocket, channels involved in nociception, were dispensable for cephalic touch sensation (Fig. 1B).

The phenotype of the *nompC* mutant paralleled the previous findings on the role of *nompC* in gentle touch [2]. To gain mechanistic insights onto how nompC senses head touch, we first explored its expression in the cephalic segment with nompC driver lines. There are four types of external sensory organ in the larval head: the terminal organ (TO), dorsal organ (DO), ventral organ (VO), and labial organ [9]. In the DO, nompC-QF labels neurons in a non-overlapping pattern with Gr21a- and Or83b- positive olfactory neurons (Fig. 1F), suggesting that these neurons are tubular body-containing neurons in the cylindrical portion of the DO [10, 11]. In the TO, nompC^{GFP} knock-in exhibited regular sensilla distribution and sub-cellular

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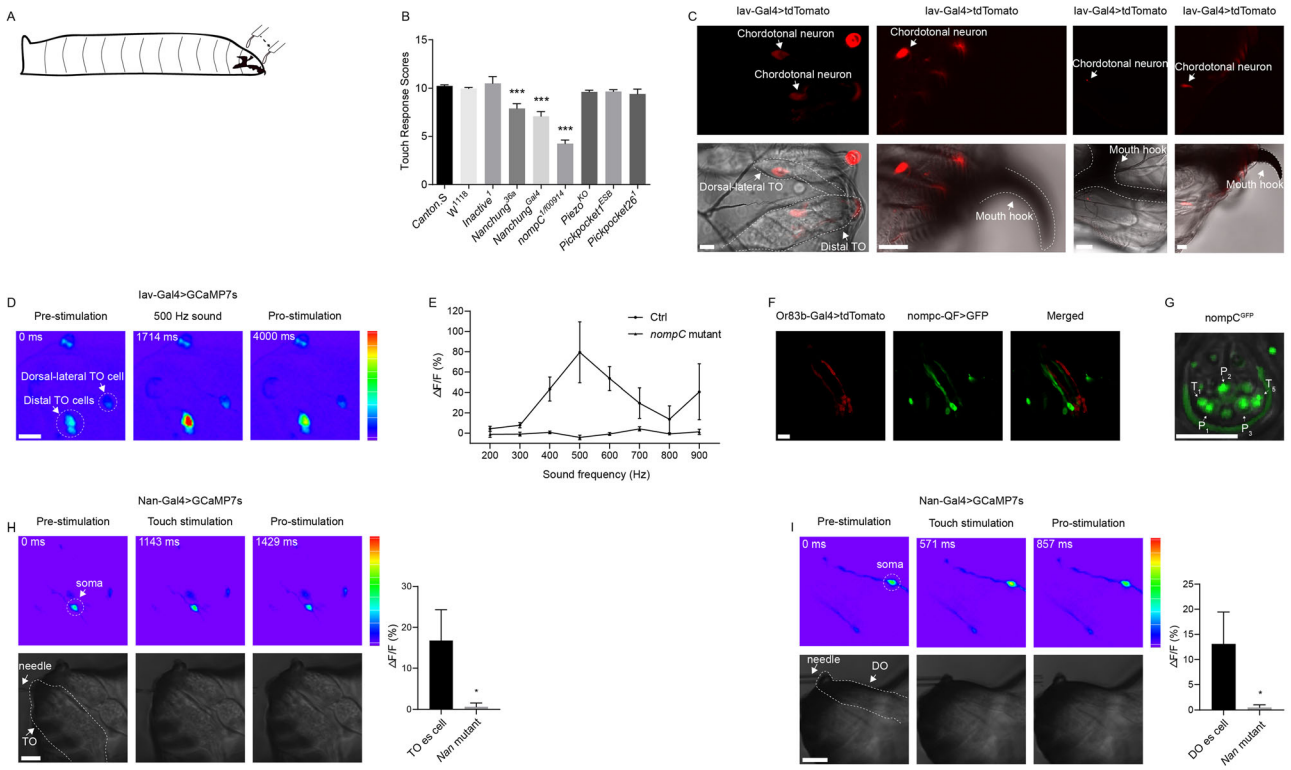


Fig. 1 TRP channel genes are required for larval cephalic mechanosensation. **A** Schematic of the larval head-touch assay. Gentle touch stimuli were applied to the head from posterior to anterior. **B** Screen for the head-touch response in mutants of candidate mechanotransduction channel genes. Mutants of the TRPV gene nanchung show a defective response. **C** Iav-Gal4 marks chordotonal neurons in the larval head, From left to right: two cells (white arrowheads) in the TO with cell bodies located in the TOG; one cell (white arrowhead) in the dorso-distal part of the TO with the cell body located in the "bridge" between DO and TO; one cell located under mouthhook (white arrowhead); one cell may belong to the thoracic internal organ (white arrowhead); and one cell located around the mouth (white arrowhead) (scale bars, 10 μm). **D** Cephalic chordotonal neurons (labelled by both iav and nan) respond to 500 Hz sound stimuli. Genotype is iav-Gal4; UAS-GCaMP7s (color range, 0–255; scale bar, 10 μm). **E** Chordotonal neurons in the terminal organ have a frequency selectivity similar to abdominal chordotonal neurons. The response to sound disappears in the nompC mutant. Ctrl: w; iav-Gal4/+; UAS-GCaMP7s/+. nompC mutant: nompC^{1}/nompC^{3}. **F** nompC-QF (green) labels neurons in the dorsal organ. Olfactory neurons are labelled with the Or83b-Gal4. Green: w; nompC-QF, QUAS-GFP. Red: or83b-Gal4, UAS-tdTomato (scale bar, 10 μm). **G** nompC is enriched in the tip of the TO sensilla (anterior view; scale bar, 10 μm). **H**, **I** Cephalic es neurons in the TO (**H**) and DO (**I**) from wild-type and *Nan^{Gal4}* flies respond to external mechanical stimuli (color range, 0–255; scale bars, 10 μm). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$

localization. We saw strong GFP signals from the dendrite tips, indicating a highly specialized subcellular enrichment of the nompC protein (Fig. 1G).

Since the *nan* mutant also exhibited a significantly reduced head-touch response (Fig. 1B), we next asked how nan functions in touch sensation. We first checked the expression of nan-Gal4 in the larval head sensory organs. Nine neurons were labeled in each hemi-segment and 6 of them were co-labelled with iav-Gal4. Nan and iav are usually co-expressed in chordotonal neurons that sense stretch and vibration [6, 12]. Among these 6 neurons, one was located in thoracic internal organs, one under the mouth hook, one in the mouth that may belong to the labial organ, two in the TO, and one in the dorsolateral part of the TO (Fig. 1C). The larval Cho neurons are vibration sensors that trigger an avoidance response [6]. All 6 of these neurons showed a significant response to 500 Hz sound stimuli (data not shown). We also checked the frequency-dependence of the Cho neurons in the TO. These two neurons showed a frequency dependence similar to abdominal Cho neurons (Fig. 1E). We thus speculated that the cephalic Cho neurons play a role in vibration detection in the cephalic segment, although we were not able to test their functional involvement as there is no specific marker for these neurons.

In contrast, the 3 nan^{+}iav^{-} neurons were separately located in the TO, DO, and VO. The neuron in TO may belong to the T_1 sensilla that contained a tubular body-positive neuron (data not shown). We then checked the co-expression of nan and nompC in the TO and DO, and found that, compared to nompC-QF larvae, the average number of neurons expressing GFP did not change when combining

nan-Gal4 and nompC-QF, indicating that the nan-Gal4-labeled neurons in the TO and DO also express nompC. Based on their morphology, these $nan^{+}nompC^{+}iav^{-}$ neurons were likely es organ neurons. As described above, *nan* mutant larvae showed a reduced touch response while *iav* mutants did not, suggesting that the es organ neurons in the TO and DO are essential for touch sensation. To test this hypothesis, we monitored the activity of the nan^{+} es organ neurons with the Ca^{2+} indicator GCaMP [6]. These neurons responded to touch on the cephalic end and the response was largely absent from the *nan* mutant larvae (Fig. 1H, I). The Nan-Gal4 used here did not label other external organ or md neurons in the body segments [12], indicating that the cephalic es organs may be enriched in specific mechanoreceptors.

Mechanosensation in the cephalic segments exhibits features remarkably different from that in the other body segments. To gain more insight into how the spatial pattern of gene expression contributes to this inter-segmental heterogeneity, we used RNA-seq to identify genes enriched in the larval head. We collected mRNA from the cephalic segment and analyzed the differential gene expression relative to the A4–A6 abdominal segments.

To verify that our RNA-seq faithfully identified genes with higher expression in the head, we used the Berkeley *Drosophila* Genome Project *in situ* database (https://insitu.fruitfly.org/cgi-bin/ex/insitu.pl), Flymine (http://www.flymine.org/flymine/begin.do), and Flybase (http://flybase.org/) to annotate the expression patterns and molecular functions of the top 40 genes from our RNA-seq results (Table S1), some of which were reported to be expressed in the chemosensory and visual organs located on the head. Most of the rest were expressed in the embryonic head epidermis, embryonic antennal sensory organs, and other organs in the head. These results validated the reliability of our RNA-seq data.

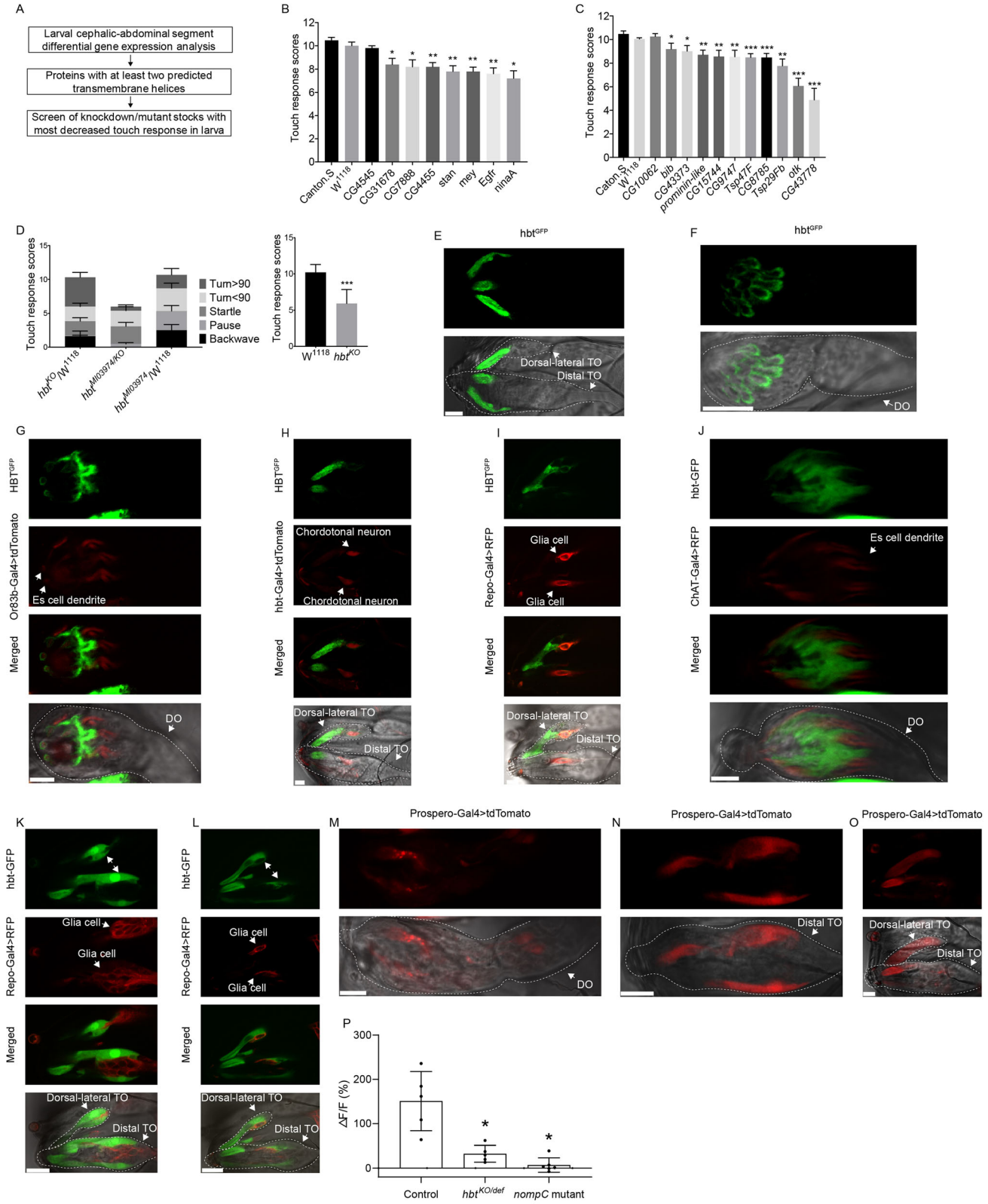
To begin discerning the molecular basis of the heterogeneity of mechanosensation in the larval cephalic segments, we first examined the genes with trans-membrane domains that are most likely involved in mechanotransduction. We selected those with at least two predicted trans-membrane domains from the cephalically-enriched genes in our RNA-seq, resulting in a pool of 146 genes as candidates for our initial behavioral screening (Fig. 2A). We obtained RNAi and/or mutant stocks for most of these genes and tested the head-touch response. Among the 17 genes that showed reduced touch sensitivity (Fig. 2B, C), some have been reported to be important for the development of *Drosophila* md neurons [13], so it thus was surprising that interference with their function disrupted the touch response. For example, starry night (stan) is one of the two adhesion G-protein-coupled receptors in fly because its extracellular domain has cell-cell adhesion motifs [14]. In *stan* mutant embryos, md neurons show overextended dendrites [13], which may account for the reduced gentle touch response found here. Another example is Otk (Fig. 2B), a gene required for the projection of motoneuron axon tracts [15, 16]. Both motor and sensory problems could contribute to the touch defect of the *otk* mutant.

Among all these hits, a previously uncharacterized gene, CG43778, was of particular interest because its mutant had the most severe touch defect among all the genes tested and its molecular/cellular function had not yet been studied (Fig. 2B). We named this gene headbutt (hbt). The MIMIC (Minos-mediated integration cassette) insertion of HBT showed only half of the touch response score of the wild-type w^{1118} (Fig. 2C). To confirm this touch response phenotype, we generated a full knockout of HBT using the CRISPR/Cas9 method. The back-crossed homozygous knockout of HBT and the trans-heterozygotes of MIMIC and knockout showed a touch response defect as severe as the MIMIC line (Fig. 2D), indicating that this gene is critical for mediating the head-touch response.

To further investigate how HBT is involved in touch sensation, we knocked-in a superfolder GFP at the C-terminal of the HBT coding sequence (HBT^{GFP}). The GFP fluorescence then served as an indicator for the distribution HBT protein. We found strong GFP signals in the TO and DO (Fig. 2E, F). In the TO, the expression of HBT was divided into dorsolateral and distal parts (Fig. 2E). Interestingly, HBT protein was notably enriched in the TO but not the TOG (terminal organ ganglion) (Fig. 2E), forming a tubule-like structure in the dorsolateral part that hosts the cell bodies of the sensory neurons (Fig. 2F). We also observed that HBT protein forms a sheath-like layer surrounding the brain and the ventral nerve cord, a structure resembling glial ensheathment [17]. In the DO, HBT^{GFP} formed a tube-like structure (Fig. 2F). We checked the co-localization of HBT^{GFP} cells and Or83b chemosensory neurons and found that, like the Cho neurons in the TO, Or83b-labeled sensory neurons were enwrapped by HBT^{GFP} cells (Fig. 2G).

The MIMIC line for hbt used here was generated by inserting a MiMIC transposon in the first intron after a non-coding exon in the HBT genome region. The MiMIC transposon contains an enhanced GFP after the three stop codons but before the ATG of the HBT coding sequence, thus resulting in the production of GFP under the control of the regulatory regions of HBT and reflecting the expression pattern of HBT (we named this line hbt-GFP as it used a mechanism similar to enhancer-trap). Indeed, the GFP expression driven by this hbt-GFP largely recapitulated the pattern of the HBT^{GFP} larvae (Fig. 2J–L).

We next asked how HBT regulates mechanosensation as an extrinsic protein. During the development of each type I


A
Larval cephalic-abdominal segment differential gene expression analysis
Proteins with at least two predicted transmembrane helices
Screen of knockdown/mutant stocks with most decreased touch response in larva
B
Touch response scores
Canton S
W1118
CG4545
CG31678
CG7888
CG4455
stan
mey
Egfr
ninaA
C
Touch response scores
Caton S
W1118
CG10062
bib
CG43373
prominin-like
CG15744
CG9747
Tsp47F
CG8785
Tsp29Fb
otk
CG43778
D
Touch response scores
Turn>90
Turn<90
Startle
Pause
Backwave
hbtKO/W1118
hbtMI03974/KO
hbtMI03974/W1118
W1118
hbtKO
E
hbtGFP
Dorsal-lateral TO
Distal TO
F
hbtGFP
DO
G
HBTGFP
Or83b-Gal4>tdTomato
Es cell dendrite
Merged
DO
H
HBTGFP
hbt-Gal4>tdTomato
Chordotonal neuron
Chordotonal neuron
Merged
Dorsal-lateral TO
Distal TO
I
HBTGFP
Repo-Gal4>RFP
Glia cell
Glia cell
Merged
Dorsal-lateral TO
Distal TO
J
hbt-GFP
ChAT-Gal4>RFP
Es cell dendrite
Merged
DO
K
hbt-GFP
Repo-Gal4>RFP
Glia cell
Glia cell
Merged
Dorsal-lateral TO
Distal TO
L
hbt-GFP
Repo-Gal4>RFP
Glia cell
Glia cell
Merged
Dorsal-lateral TO
Distal TO
M
Prospero-Gal4>tdTomato
DO
N
Prospero-Gal4>tdTomato
Distal TO
O
Prospero-Gal4>tdTomato
Dorsal-lateral TO
Distal TO
P
ΔF/F (%)
Control
hbtKO/def
nompC mutant

◀**Fig. 2** HBT functions in sheath cells for cephalic sensory organs to sense touch. **A** Flowchart of the screen for genes involved in the larval head-touch response. **B** Hits from the screen for genes with gene-specific RNAis. UAS-RNAi lines were crossed to Cha-Gal4 ($n \geq 5$); CG4545 is the positive control. **C** Hits from the screen using mutant alleles ($n \geq 10$); CG10062 is the positive control. **D** Gentle touch response of *hbt* mutant larvae ($hbt^{MI03974}$, HBT MiMIC insertion; hbt^{KO}, HBT full knockout). **E** HBT is enriched in the anterior region of both the dorsolateral and distal parts of the TO (scale bar, 10 μm). **F** HBT proteins bundle to form a tube-like structure in the DO (scale bar, 10 μm). **G** In the DO, HBT proteins wrap the dendrites of es cells (green, HBT^{GFP}; red, Or83b > tdTomato; scale bar, 10 μm). **H** In the TO, HBT proteins wrap the cell bodies of chordotonal neurons (green, HBT^{GFP}; red, chordotonal neurons, HBT-Gal4 > tdTomato; scale bar, 10 μm). **I** In the TO, HBT proteins wrap glial cells of chordotonal neurons (green, HBT^{GFP}; red, glial cells, Repo-Gal4 > RFP; scale bar, 10 μm). **J–L** HBT MiMIC labels cells in a pattern similar to HBT^{GFP} in the DO (**J**), TO (**K**), and glial cells ensheathing chordotonal neurons (**L**) (green, HBT MiMIC insertion line; red, Repo-Gal4 > RFP (**J**), ChAT-Gal4 > RFP (**K**, **L**); scale bars, 10 μm). **M–O** prospero-Gal4 labels sheath cells in the DO, distal TO, and dorsolateral TO (red, prospero-Gal4 > UAS-tdTomato; scale bars, 10 μm). **P** HBT is required for larval TO mechanosensation. *hbt* mutant larval chordotonal neurons in the TO show a significantly reduced Ca^{2+} response (*nompC* mutant is the positive control). $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$

sensilla, four asymmetric mitotic divisions produce one or more sensory neurons, three surrounding support cells, and a glial cell [18]. The thecogen cell forms the sheath cell of neurons, may secret the extracellular matrix surrounding the neural dendrites [18], and can be labeled by the transcription factor prospero. The remaining glial cells accumulate the common glia marker Repo. We first checked whether the hbt-expressing cells are indeed glial cells. We used Repo-Gal4 to express RFP in glial cells in larval TOs and DOs. A glial cell was found in the dorsal-distal part of the TO where one chordotonal neuron soma is located, and a ring-like structure surrounds this soma (Fig. 2H, I). The cells labeled in the hbt-GFP line also formed a large ring-like structure that exactly surrounded the glial cell (Fig. 2L). The other part of this cell extended to the distal part of the TO like HBT^{GFP}. The distal part of the TO contained two chordotonal neurons whose cell bodies were located more distal in the TOG (Fig. 2L), although the glial cell was larger.

In the DO, however, the situation was slightly different. The DO does not contain any chordotonal neurons and all cell bodies are located in the DOG (dorsal organ ganglion). Glial cells were only found in the proximal part of the DO and they did not entirely ensheath the dendrites of the DO sensory neurons. Instead, the HBT-GFP cells were not located in the DOG, but they formed a tube-like structure in the DO similar to HBT^{GFP} (Fig. 2J). This was reminiscent of the prospero-positive sheath cells of the DO [19]. Previous work has suggested that the DO is composed of 14 sensilla, each containing a sheath cell [19]. Considering the very similar structure, we reasoned that HBT is expressed in sheath cells in the type I sensilla of the DO and TO. We used the enhancer-trap line of prospero to drive expression of tdTomato. Importantly, the expression patterns in the TO and DO were similar to those of HBT protein (Fig. 2M–O). These results support the hypothesis that HBT is expressed in sheath cells of the TO and DO.

The larval TO and DO both contain mechanosensory type I cells. As *hbt* mutant larvae show a much-reduced touch response and HBT is enriched in the distal part of the sheath cells, we next asked whether *hbt* mutation influences the mechanosensitivity of type I neurons in the TO and DO. We tested whether hbt is required for the Ca^{2+} response of the TO chordotonal neurons and found that, compared to the control group, TO Cho neurons in HBT mutant larvae had a reduced Ca^{2+} response to touch (Fig. 2P). As a positive control, we found that in the *nompC* mutant larvae the Cho neurons lost their normal response to external mechanical stimuli (Fig. 2P), similar to the abdominal Cho neurons [6].

HBT does not have known conserved orthologues in vertebrates. Although this gene appears to play a very specific role in sheath cells in the TO and DO, its molecular function is still an open question. HBT protein is enriched in the anterior parts of both the TO and DO. Given that it has four transmembrane domains and forms a thin tubular structure in the DO, it is possible that HBT is a transmembrane protein and is required for the normal connections between hbt-expressing cells and other parts of the DO and TO; it is also conceivable that hbt protein is important for maintaining a stable lumen micro-environment in the TO and DO. However, due to the lack of molecular motif prediction, the molecular roles of HBT protein remains to be investigated.

In summary, we characterized the mechanosensory neurons in the larval cephalic segments and found that a triad of TRP channels mediate their mechanosensitivity. Among these, Nan and Iav were co-expressed in chordotonal neurons in the TO, thoracic internal organs, and a chordotonal neuron underneath the mouth hook. Besides, Nan was expressed in three external sensory organ neurons in the TO, DO, and VO and these neurons were also nompC-positive. Both nan and nompC were required for sensing touch on the larval head. Furthermore, by combining RNA-seq and behavioral screening, we identified the novel gene headbutt (hbt) that functions in the cells surrounding the DO and TO to regulate the neuronal mechanosensitivity. Taken together, our study demonstrates that the transduction of mechanical force requires a complete cohort of cellular and molecular machinery and provides an entry point to investigate the neural basis of

cephalic mechanotransduction and inter-segmental mechanosensory integration in *Drosophila*.

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