Title: Proprioceptive Opsin Functions in *Drosophila* Larval Locomotion

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Running title: Opsins in proprioception

SUMMARY

Animals rely on mechanosensory feedback from proprioceptors to control locomotory body movements. Unexpectedly, we found that this movement control requires visual opsins. Disrupting the *Drosophila* opsins NINAE or Rh6 impaired larval locomotion and body contractions, independently of light and vision. The opsins were detected in chordotonal proprioceptors along the larval body, localizing to their ciliated dendrites. Loss of opsins impaired mechanically-evoked proprioceptor spiking and cilium ultrastructure. Without NINAE or Rh6, NOMPC mechanotransduction channels leaked from proprioceptor cilia and

ciliary Inactive (Iav) channels partly disappeared. Locomotion is shown to require opsins in proprioceptors, and the receptors are found to express the opsin gene *Rh7*, in addition to *ninaE* and *Rh6*. Besides implicating opsins in movement control, this documents roles of non-ciliary, rhabdomeric opsins in cilium organization, providing a model for a key transition in opsin evolution and suggesting that rhabdomeric opsin functions in dendrite maintenance evolutionarily preceded their use for light detection.

Key words: chordotonal organ, cilium maintenance, Inactive channel, locomotion, mechanosensory transduction, movement control, Nanchung, NOMPC, proprioception, rhabdomeric opsin evolution, sensory dendrite organization, TRP cation channel.

HIGHLIGHTS:

• *Drosophila* larval locomotion requires the visual opsins NINAE and Rh6

• NINAE and Rh6 occur in ciliated proprioceptor dendrites along the larval body

• Loss of NINAE or Rh6 impairs proprioceptor function and TRP channel localization

• NINAE and Rh6, though rhabdomeric, contribute to cilium integrity.

INTRODUCTION

Work on *Drosophila* has shown that opsins, the protein moieties of visual photopigments, not only detect light (Leung and Montell, 2017). Opsins were implicated in larval temperature preference behaviors (Shen *et al.*, 2011; Sokabe *et al.*, 2016) and in hearing in the adult flies (Senthilan *et al.*, 2012). Accordingly, opsins were surmised to have light-independent ancestral functions (Shen *et al.*, 2011; Senthilan *et al.*, 2012), yet what these functions are remains a mystery. In thermosensation, opsins, though usually thermo-stable, were proposed to act as

thermosensors (Shen *et al.*, 2011; Leung and Montell, 2017), and how opsins contribute sound transduction is unclear (Albert and Göpfert, 2015; Leung and Montell, 2017). Locomotion is a hallmark of animal life, and the need for movement control presumably was a driving factor in early nervous system evolution (Jékely *et al.*, 2015). Key components in locomotion control are proprioceptors that inform the nervous system about locomotory body movements (Büschges and El Manira, 1989). *A priori*, we could not expect that proprioceptors would require visual opsins, but when we analyzed locomotion in *Drosophila* larvae, this is what we found.

RESULTS

To assay locomotion, we recorded late 3rd instar wandering larvae crawling singly in transparent arenas. Because visual input can influence locomotion (Busto *et al.*, 1999), all experiments were carried out under infrared illumination that does not activate *Drosophila* opsins (Salcedo *et al.*, 1999; Ni *et al.*, 2017). Notwithstanding this exclusion of visual effects, $ninaE^{17}$ null mutant larvae lacking the major visual opsin NINAE (also known as Rh1) displayed defective locomotion (Fig. 1A, see also Supplemental movie S1). Compared to wild-type larvae, $ninaE^{17}$ mutants crawled slower and turned more frequently, requiring more time to travel by one body length and covering shorter distances between consecutive turns (Figs. 1B and S1A). Equivalent locomotion defects ensued when the $ninaE^{17}$ mutation was uncovered with the deficiency *Df(3R)BSC636*, whereas normal locomotion was restored when we introduced a genomic *ninaE* rescue construct, $P\{ninaE^{+}\}$, into the $ninaE^{17}$ mutant background (Figs. 1A,B, S1A, and movie S1). The requirement of NINAE for locomotion cannot reflect visual *ninaE* function; NINAE, the major visual opins of adult flies, is absent from larval photoreceptors, which instead use the minor opsins Rh5 or Rh6 (Sprecher *et al.* 2007). To evaluate whether larval crawling might also depend on other opsins, we randomly selected $Rh6^{1}$ null mutants lacking the Rh6 opsin, revealing locomotion phenotypes as seen in $ninaE^{17}$ mutants (Figs. 1B, S1A). Locomotion defects were also seen when the $Rh6^{1}$ mutation was uncovered with the deficiency

Df(3R)Exel6174, and introducing a genomic *Rh6* rescue construct, *P{Rh6^{+}}*, in the *Rh6^{1}* mutant background restored normal locomotion (Figs. 1B, S1A).

Impaired locomotion in *ninaE^{17}* mutants was associated with altered locomotory body movements. Compared to controls, the mutant larvae pushed forward their bodies with diminished contraction amplitudes (Figs. 1C, S1B). These movement defects were largely ameliorated in *ninaE^{17}, P{ninaE^{+}}* rescue larvae, and contraction defects were also seen in *Rh6^{1}* mutants where they were rescued fully by the rescue construct *P{Rh6^{+}}* (Figs. 1C, S1B).

Locomotion defects as seen in *ninaE^{17}* and *Rh6^{1}* mutants reportedly characterize mutant larvae with impaired chordotonal organs (Caldwell *et al.*, 2003). Chordotonal organs are internal stretch receptors that are serially arranged along the insect body. Besides monitoring substrate vibrations, chordotonal organs primarily function as proprioceptors controlling locomotion (Field and Matheson, 1999). We assayed locomotion in *nanchung* (*nan*) and *inactive* (*iav*) ion channel mutants with silenced chordotonal organs (Zhang *et al.*, 2013), revealing pronounced locomotion and contraction defects resembling qualitatively those of *ninaE^{17}* and *Rh6^{1}* mutants (Figs. 1B,C, S1A,B). To test whether chordotonal organs might express opsins, we used *Rh6-Gal4* and *ninaE-Gal4* promoter/enhancer fusions to drive a hexameric *UAS-6xGFP* green fluorescent protein (GFP) reporter. *ninaE-Gal4* and *Rh6-Gal4* both induced faint GFP signals in the lateral chordotonal (lch5) organs that are serially arranged along the larval abdomen (Figs. 2A, S2A). Lch5 organs each comprise supporting cells and five monodendritic proprioceptor neurons. Labelling neurons with anti-horseradish peroxidase (HRP) antibody located the GFP signals to the latter proprioceptors (Figs. 2A, S2A). Additional evidence for the presence of opsins in lch5 proprioceptors was obtained when we labelled the larval abdomen with antibodies against NINAE or Rh6. Both anti-NINAE and anti-Rh6 selectively labelled the sensory dendrites of all lch5 receptors in wild-type larvae but not in the respective null mutants (Figs. 2B,C and S2B). Chordotonal receptor dendrites use cilia as outer segments, with each cilium being surrounded by an endolymph space (Field and Matheson, 1998). Anti-HRP recognizes sugar residues of glycoproteins that are transported up into these cilia where they are secreted in two bands into that space (Jan *et al.*, 1985). The first HRP band

demarks the junction between the dendritic inner segment and the cilium. Both anti-NINAE and anti-Rh6 selectively labelled the dendritic inner segment, up to this first HRP band (Figs. 2B,C and S2B). Within the dendritic inner segment, anti-Rh6 staining was confined to the most distal region, whereas anti-NINAE staining extended further down the segment (Figs S2B,C and S2B), indicating partly different localizations for the two opsin proteins.

Chordotonal receptor cilia transduce mechanical stimuli that are conveyed to them by supporting cap cells (Field and Matheson, 1998). To test whether opsins contribute to chordotonal receptor function, we recorded compound action currents from lch5 receptor axons while displacing the cap cells with 20 Hz sinusoids (Fig. 3A). Consistent with previous observations (Zhang *et al.*, 2013), wild-type lch5 receptors spiked spontaneously and increased their spike rate during cap cell actuation (Fig. 3B,C). Spontaneous spiking was preserved in $ninaE^{17}$ and also $Rh6^{1}$ mutants, (Fig. 3B,D), yet the spike rate hardly increased in response to mechanical stimulation (Fig. 3B,C). This reduced responsiveness was seen across a wide range of stimulus displacements (Fig. 3C), including large stimuli evoking saturated spiking (Fig. 3E). Virtually normal spiking responses were restored in both $ninaE^{17}$, $P\{ninaE^{+}\}$ and $Rh6^{1}$, $P\{Rh6^{+}\}$ rescue larvae (Fig. 3B,C,E), documenting that the mechanosensory function of the proprioceptors depends on opsins.

Mechanotransduction by lch5 receptors involves the ciliary transient receptor potential (TRP) channels NOMPC, Nanchung (Nan), and Inactive (Iav) (Zhang et al., 2013). NOMPC mechanotransduction channels (Yan *et al.*, 2013) localize distally of the second HRP band to the tip of the cilium (Cheng *et al.*, 2010), whereas Nan and Iav form heteromeric channels in the proximal cilium region, between the two HRP bands (Gong *et al.*, 2004; Nesterov *et al.*, 2015) (Fig. 4A). We co-stained lch5 receptors with antibodies against NOMPC and Iav and found that the ciliary localization of these channels depends on opsins. Wild-type lch5 cilia showed normal NOMPC and Iav localizations, with Iav occurring proximally to NOMPC (Figs. 4A, S3). In $ninaE^{17}$ mutants, however, NOMPC leaked into the endolymph space from about half of the cilia, whereby some of the affected cilia lacked Iav protein (Fig. 4B,D). Ciliary leakage of NOMPC, and an associated loss of Iav, was also seen when we selectively knocked

down *ninaE* in chordotonal receptors by expressing a *UAS-ninaE-RNAi* construct with the chordotonal receptor driver *dnai2-Gal4* (Karak *et al.*, 2015) (Fig. 4C,D). Control larvae expressing either *ninaE-RNAi* or *dnai2-Gal4* alone showed largely normal TRP channel localizations, and, in $ninaE^{17}$ mutants, the ciliary defects were attenuated by the rescue construct $P\{ninaE^{+}\}$ (Fig. 4C,D). NOMPC leakage and loss of IAV also characterized the lch5 receptors of $Rh6^{1}$ mutants, where the rescue construct $P\{Rh6^{+}\}$ restored partially cilium integrity (Fig. 4C,D). Defects in cell integrity were also seen when we inspected lch5 receptor dendrites in $ninaE^{17}$ mutants with transmission electron microscopy (Fig. 4E-G). Besides seeminly intact dendrites (Fig. 4E,F), we observed profound structural aberrations in the mutants, including cilium bending (Fig. 4G, left), signs of cilium blebbing and disorganization (Fig. 4G, middle), and entire cilium loss with cell material invading the endolymph space, including the electron dense material that normally resides in a dilation of the cilium (Fig. 4G, right).

That locomotion requires feedback from chordotonal receptors was confirmed when we selectively ablated these receptors by expressing *UAS-hid,rpr* with the chordotonal receptor driver *Dhc93AB-Gal4* (Senthilan *et al.*, 2012). Successful ablation was reported by anti-HRP staining (Fig. S4G), and locomotion was found to be impaired (Fig. S4A). Locomotion defects, though less pronounced, also ensued when we used RNAi to selectively knock down *ninaE* in chordotonal receptors (Fig. S4B), documenting that these proprioceptors render locomotion dependent on opsin. Locomotion defects in $ninaE^{17}$, $Rh6^{1}$ double mutants resembled those of the single mutants (Fig. S4C), indicating that NINAE and Rh6 operate in the same pathway, with both being essential for normal locomotion. To evaluate whether also the opsin-bound retinal chromophore might be required for larval crawling, we analysed locomotion $santa\text{-}maria^{1}$ mutants that fail to synthesize the chromophore (Wang *et al.*, 2007). $santa\text{-}maria^{1}$ mutants showed locomotion defects resembling those of $ninaE^{17}$ and $Rh6^{1}$ mutants (Fig. S4D), indicating that proprioceptive opsin functions might involve the chromophore. The opsin-dependence of larval temperature preferences had been linked to neurons that express the TRPA1 channel (Shen *et al.*, 2011; Sokabe *et al.*, 2016). This channel, however, is reportedly absent from chordotonal receptors (Rosenzweig *et al.*, 2005), and locomotion remained largely

uncompromised when we ablated TRPA1-positive neurons by driving *UAS-hid,rpr* with *trpA1-Gal4* (Fig. S4E) To further test whether thermosensory opsin functions might affect locomotion, we assayed larval crawling at an elevated temperature of 29°C, where larval temperature behaviour is no more opsin-dependent (Shen *et al.*, 2011; Sokabe *et al.*, 2016). Notwithstanding this exclusion of thermosensory opsin effects, $ninaE^{17}$ and $Rh6^{1}$ mutants displayed defective locomotion (Fig. S4F), documenting that the proprioceptive functions of opsins are independent of their thermosensory roles.

DISCUSSION

Apart from photoreceptors, chordotonal proprioceptors seem to be the first animal cells whose structure and function both depend on opsins. The *Drosophila* larval body wall also harbours light-sensitive cells (Xiang *et al.*, 2010), yet chordotonal receptors seems independent of light (Scholz et al., 2017) and the latter cells detect light without opsins (Xiang *et al.*, 2010). Because all *Drosophila* opsins are non-ciliary, rhabdomeric r-opsins (Shichida and Matsuyama, 2009), their presence in ciliated cells is unexpected. The co-requirements of NINAE and Rh6 seem likewise remarkable; firstly, these two opsins associate with different photoreceptor types (Sprecher *et al.*, 2007) and, secondly, even further opsins seem present in lch5 proprioceptors – judged from an intragenic *Gal4* insertion they also express the opsin *Rh7* (Fig. S2C). The ciliary leakage of NOMPC and ultrastructural defects implicate opsins in sensory dendrite organization, presumably contributing to dendrite maintenance as some dendritic cilia seem unaffected whereas others also lack the Iav protein and structurally deteriorate or disappear. Such contribution of opsins to dendrite maintenance does not exclude a sensor function as known from vision and proposed for thermosensation (Shen *et al.*, 2011). For lch5 receptors, however, a sensor seems rather unlikely for two reasons. Firstly, cap cell actuation can elicit lch5 receptor spiking within only two milliseconds (Fig. S4H), leaving no time for an indirect transduction involving upstream sensors and second messenger cascades (Christensen and Corey, 2007). Secondly, mechanotransduction in lch5 receptors takes place in the cilia, but NINAE and Rh6 localize beneath the cilia to the dendritic inner segments. The latter segments

harbour the ciliary rootlets, which support the cilia structurally and are essential for cilium maintenance (Yang *et al.*, 2005).

Evolutionarily, it now seems that fly hearing has taken over its opsin dependence from proprioception given that insect hearing organs have differentiated locally from the serial set of proprioceptive chordotonal organs (Göpfert and Hennig, 2016). The respective proprioceptors appear to be serial sibling cells of insect photoreceptors (Niwa *et al.*, 2004), suggesting that their common ancestor cells already used opsins, presumably in the context of dendrite maintenance as also the maintenance of photoreceptor rhabdomeres involves structural roles of opsins (Ahmad *et al.*, 2007; Shieh, 2011). Rhabdomeric r-opsins, as found in *Drosophila*, presumably split from ciliary c-opsins before the appearance of bilaterians (Feuda *et al.*, 2012; Held Jr., 2017), indicating that r-opsins had to step down from cilia before they were integrated into rhabdomeres (Held Jr., 2017). The dendritic localization of NINAE beneath chordotonal receptor cilia might reflect this evolutionary transition, suggesting that structural roles of r-opsins in sensory dendrites preceded their use for light detection. How exactly opsins contribute to dendrite structure is unclear. Possibly, they help shaping dendritic membrane composition given that opsins reportedly translocate phospholipids between the two membrane leaflets (Menon *et al.*, 2011). Judged from *Drosophila* photo- and chordotonal receptors, it appears that structural r-opsin functions might have been preserved during the sensory receptor cell type evolution, which could explain why diverse sensory modalities depend on opsins.

EXPERIMENTAL PROCEDURES

Animals

Animals were maintained in accordance with German Federal regulations (license Gen.Az 501.40611/0166/501). Late 3rd instar wandering larvae were used throughout. Specific details regarding the strains used the experiments can be found in the Supplementary Experimental Procedures.

Behavior

Locomotion was analyzed essentially using established methods (Risse et al., 2013). To quantify body movements, larvae were recorded while crawling on agarose gel, whereby the larvae were detected using an ellipse detection algorithm.

Antibody staining

Stainings were performed on larval fillet preparations. Details about antibodies and staining protocols are provided in the Supplementary Experimental Procedures.

Electron microscopy

Transmission electron microscopy was performed on ultrathin sections of the larval abdomen using a LEO 902 transmission electron microscope (Zeiss, Oberkochen, Germany).

Electrophysiology

Action currents were recorded with suction electrodes form the lch5 axon bundle using a HEKA (Lamprecht, Germany) patch-clamp amplifier (EPC 10 USB) under the control of Patchmaster software (HEKA) in the voltage-clamp mode. Cap cells were actuated using a piezo-actuator and controller (P-841.10 and E-709.SRG, Physik Instrumente GmbH, Karlsruhe, Germany).

Statistical Analyses

Statistical comparison of means was performed with Fisher's exact permutation tests and two-tailed Mann-Whitney U tests, using Bonferroni correction for multiple comparisons. Significance was concluded when $p < 0.05$. Data are presented as mean ± 1 SD.

AUTHOR CONTRIBUTIONS.

M.C.G. and B.G. designed research; D.G. and B.G. studied behavior with the help of D.Z. and M.A., D.Z. analyzed expression patterns and protein localization with the help of M.A. and S.P., N.S.-D. performed electron microscopy with the help of D.G. and R.K., and S.P., and B.W. analyzed physiology with the help of R.K. and S.R.. M.C.G. and B.G. wrote the manuscript together with D.Z., D.G., B.W., and N.S.-D.. All authors commented on the results and approved the manuscript.

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FIGURE LEGENDS

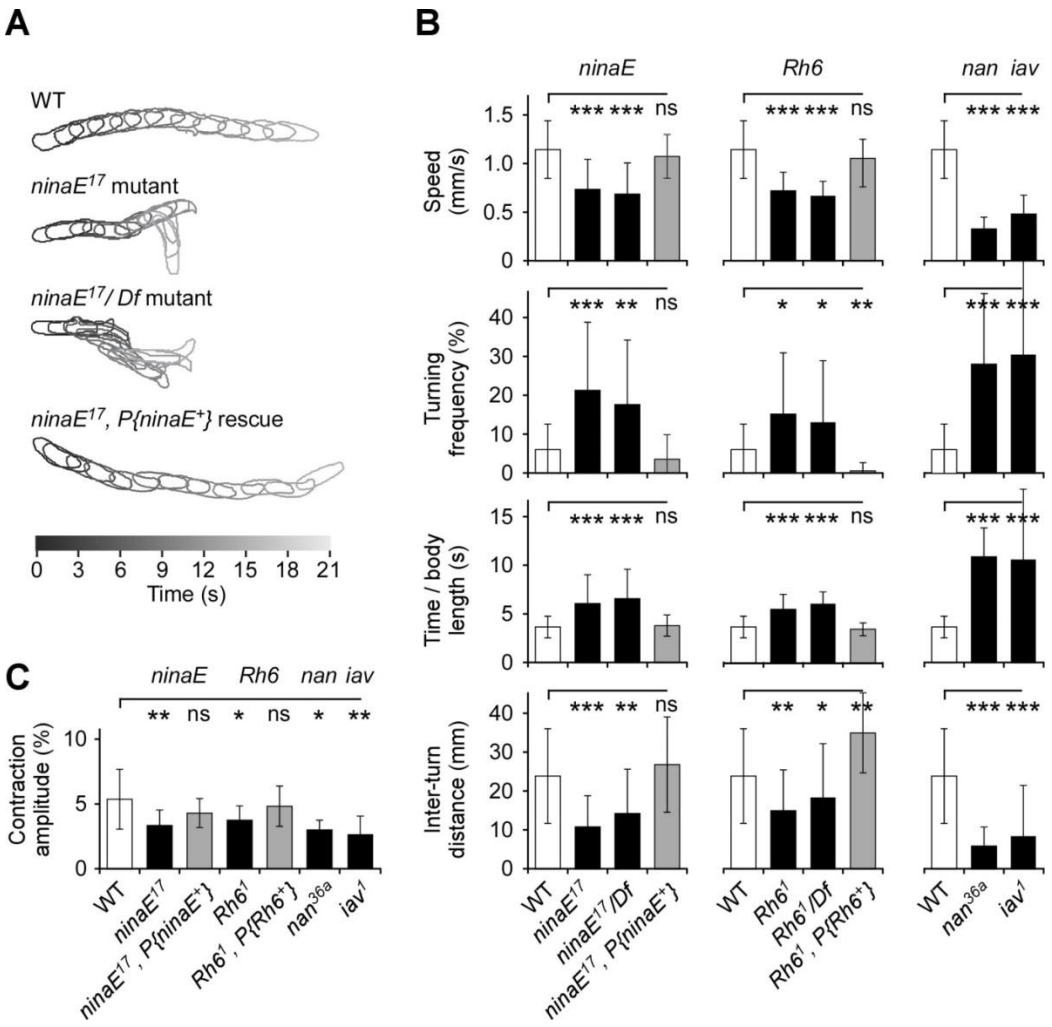

Figure 1. Locomotion requires opsin. (**A**) Example trajectories of wild-type (WT), *ninaE*^{17} and *ninaE*^{17}*/Df(3R)BSC636* (=*nina*^{17}*/Df*) mutants, and *ninaE*^{17}, *P{ninaE*$^{+}$*}* rescue larvae during 21 seconds. (**B**) Quantification of locomotion behaviour in *ninaE*^{17} and *ninaE*^{17}*/Df* as well as *Rh6*^{1} and *Rh6*^{1}*/Df* mutants, *ninaE*^{17}, *P{ninaE*$^{+}$*}* and *Rh6*^{7}, *P{Rh6*$^{+}$*}* rescue larvae, and *nan*^{36a} and *iav*^{1} ion channel mutants with silenced chordotonal receptors (means ± S.D., N = 17 to 33 larvae per strain). (**C**) Relative amplitude of locomotory body contractions normalized to individual body lengths (means ± S.D., N = 8 larvae for *nan*^{36a} and N = 12 larvae for *iav*^{1}, otherwise N = 25 to 30 larvae per strain). Bar colours in (B) and (C): white = control, black = mutant, grey = genomic rescue. Significant differences from wild-type (Fisher's exact permutation tests with Bonferroni correction): * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant. For respective comparisons against *w*^{1118} controls, see Fig. S1.

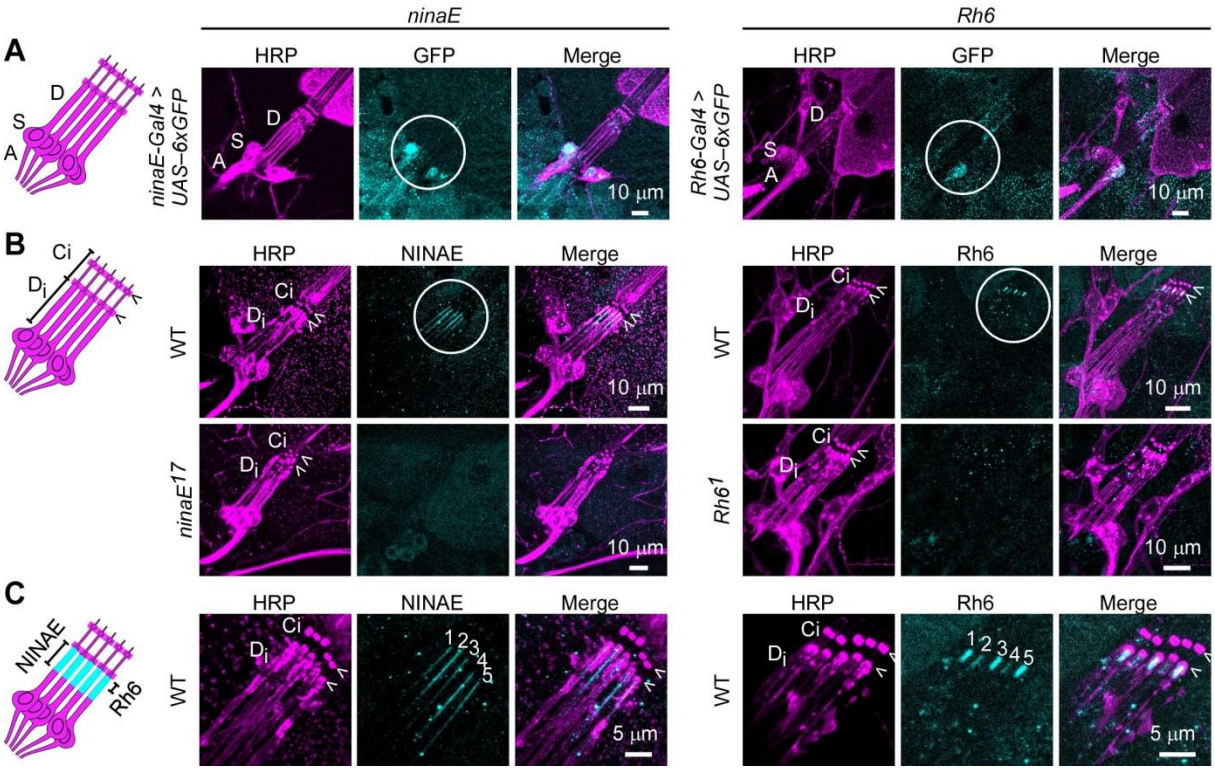

Figure 2. Opsin expression and localization in proprioceptors. (**A**) *ninaE* and *Rh6* expression in lch5. Left: sketch of lch5, depicting its five receptor cells. A = axons; S = somata; D = dendrites. Right: GFP fluorescence in lch5 receptors (white circles) obtained by driving *UAS-6xGFP* with *ninaE-Gal4* or *Rh6-Gal4*. GFP signals are enhanced with an anti-GFP antibody (cyan), and receptors are counter-stained with the neuronal marker anti-HRP (magenta). (**B**) NINAE and Rh6 localization in lch5. Left: sketch of lch5 depicting dendritic inner segments (Di), receptor cilia (Ci), and the two HRP bands (arrowheads). Both anti-NINAE and anti-Rh6 label the dendritic inner segments of lch5 receptors up to the first HRP band in wild-type (WT) larvae (top, white circles) but not in respective mutant larvae (bottom). (**C**) Zoom-ins from (B). Left: Sketch depicting NINAE and Rh6 localization. Right: Anti-NINAE and anti-Rh6 both label the dendritic inner segments of all lch5 receptors ('1-5'), whereby anti-NINAE staining extends further down along the segments. For additional examples, see Fig. S2.

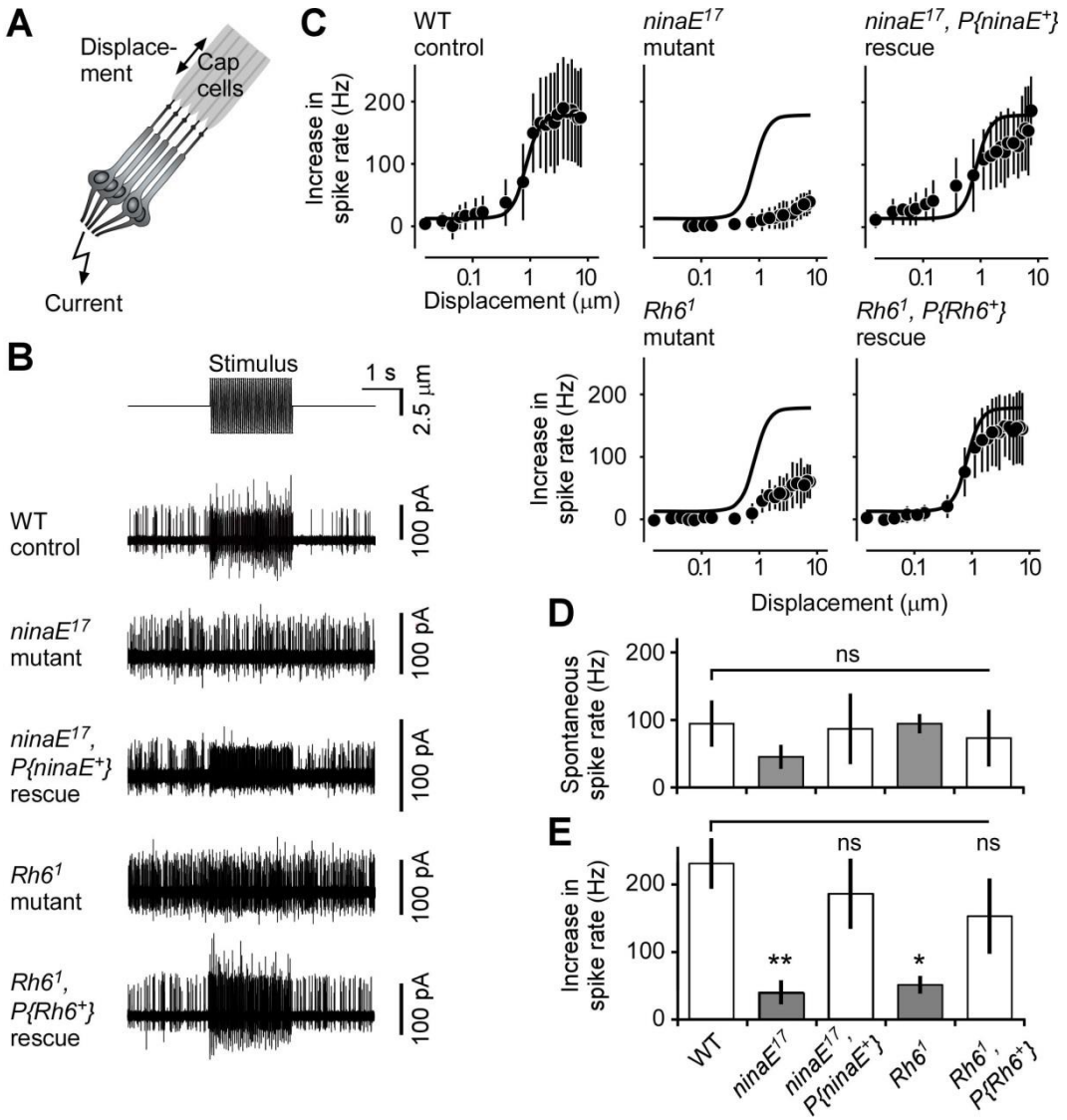

Figure 3. Proprioceptor function requires opsin. (**A**), Diagram depicting stimulation and recording sites to access mechanically evoked compound action currents of lch5 receptors. (**B**) Example traces of lch5 action currents in wild-type, mutant, and rescue larvae. Stimulus: two seconds 20 Hz sinusoid with a displacement amplitude of 2.5 µm. (**C**) Stimulus-evoked increase in spike rate as function of the stimulus displacement (N ≥ 9 animals per strain, means ± SD). Black line, repeated in each panel: Boltzmann fit to the wild-type data. For *Rh6*^{1}, *P{Rh6*$^{+}$*}* rescue larvae, the increase is more linear, possibly because the rescue is not fully complete (see panel E). (**D**) Spontaneous spike rates (N ≥ 9 animals per strain, means ± SD). (**E**) Increase in spike rate evoked by strong stimulation with 7.5 µm displacement amplitude (N ≥ 9 animals per strain, means ± SD). Asterisks: significant differences from wild-type (two-tailed Mann-Whitney U-tests with Bonferroni correction).

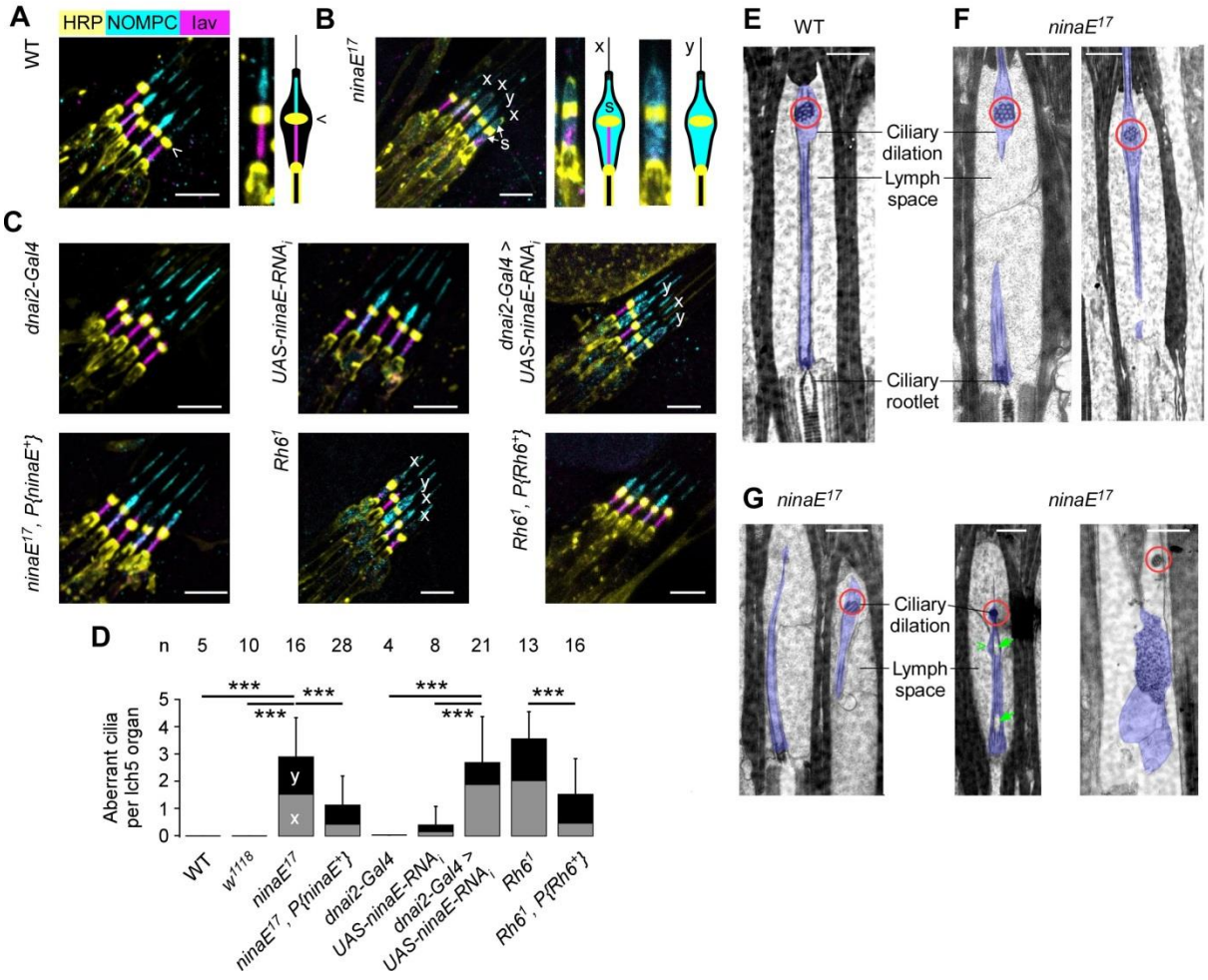

Figure 4. Opsin-dependent organization of proprioceptor dendrites. (**A-C**) Ciliary NOMPC and Iav TRP channel localization in lch5 revealed by anti-NOMPC (cyan) and anti-Iav (magenta) antibody staining. Receptors are counterstained with anti-HRP (yellow). Arrowheads: 2nd HRP band that divides the dendritic cilia into distal and proximal regions. (**A**) Wild-type (WT). NOMPC and Nan-Iav are confined to the distal and proximal cilium regions, respectively (right, zoom-in and sketch). (**B**) *ninaE*^{17} mutant. In some dendrites, NOMPC leaks into the endolymph space (s) with Iav being present ('x') in the proximal cilium region, or absent ('y'). (**C**) Respective data from *dnai2-Gal4* controls, *UAS-ninaE-RNAi* controls, *dnai2-Gal4 > UAS-ninaE-RNAi* mutants, and *ninaE*^{17}, *P{ninaE*$^{+}$*}* rescue larvae as well as *Rh6*^{1} mutants and *Rh6*^{1}, *P{ninaE*$^{+}$*}* rescue larvae. (**D**) Numbers of aberrant cilia per lch5 organ (means ± S.D.). 'x': grey, 'y': black) (n = numbers of tested lch5 organs). Asterisks: significant differences (two-tailed Mann-Whitney U-tests with Bonferroni correction). Scale bars (**A-C**): 5 µm. For additional data, see Fig. S3. (**E-G**) Transmission electron micrographs of lch5

receptor dendrites. Cilia are highlighted in blue and the ciliary dilation, the surrounding endolymph space, and the ciliary rootlets are indicated. Red circles highlight electron-dense material that fills the ciliary dilation. Scale bars: 1μm. (**E**) wild-type (WT) receptor. (**F,G**) *ninaE*^{17} mutant receptors with seemingly normal (**F**) and defective (**G**) cilia, the latter displaying cilium bending (**G**, left), signs of cilium blebbing (green arrowhead) and disorganization (green arrows) (G, middle), and complete loss of the cilium with the electron dense material of the ciliary dilation (red circle) occuring in the endolymph space (G, right). Images were taken from one wild-type lch5 organ and three *ninaE*^{17} mutant organs. For additional examples of wild-type receptors, see Scholz *et al.*, 2017.