Report

Neuron

TRP Channels in Insect Stretch Receptors as Insecticide Targets

Highlights

- Two commercial insecticides disrupt insect coordination and hearing
- The insecticides silence stretch receptors that co-express the TRPs Iav and Nan
- Nan and Iav together confer cellular insecticide responses in vivo and in vitro
- The two insecticides are specific agonists of Nan-Iav complexes

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In Brief

TRP channels form homo- and heteromers in many cell types. Nesterov et al. show that two commercial insecticides target a heteromeric TRP that is specific for insect stretch receptor cells.

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TRP Channels in Insect Stretch Receptors as Insecticide Targets

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SUMMARY

Defining the molecular targets of insecticides is crucial for assessing their selectivity and potential impact on environment and health. Two commercial insecticides are now shown to target a transient receptor potential (TRP) ion channel complex that is unique to insect stretch receptor cells. Pymetrozine and pyrifluquinazon disturbed *Drosophila* coordination and hearing by acting on chordotonal stretch receptor neurons. This action required the two TRPs Nanchung (Nan) and Inactive (Iav), which co-occur exclusively within these cells. Nan and Iav together sufficed to confer cellular insecticide responses in vivo and in vitro, and the two insecticides were identified as specific agonists of Nan-Iav complexes that, by promoting cellular calcium influx, silence the stretch receptor cells. This establishes TRPs as insecticide targets and defines specific agonists of insect TRPs. It also shows that TRPs can render insecticides cell-type selective and puts forward TRP targets to reduce side effects on non-target species.

INTRODUCTION

Most highly effective insecticides act on targets specific to insect nerves and muscles (Bloomquist, 1996; Casida, 2009; Casida and Durkin, 2013; Lümmen, 2013). Despite decades of intensive research to discover new insecticides and insecticide targets, commercial neuroactive insecticides all seem to converge on only seven molecular targets, the last of which was uncovered 30 years ago (Duce and Scott, 1985). Because neuroactive insecticides often act on ion channels, it was speculated that some insecticides might target transient receptor potential (TRP) family members (Lümmen, 2013). TRPs form homo- and heteromeric cation channels in diverse cell types (Venkatachalam and Montell, 2007), but experimental evidence demonstrating that insecticides affect insects by acting on TRPs has not been reported so far (Casida and Durkin, 2013; Lümmen, 2013).

Pymetrozine (PM) and pyrifluquinazon (PFQ) (see Figure S1A) are two commercial synthetic insecticides with unknown molecular targets (Maienfisch, 2012; Casida and Durkin, 2013). PM and PFQ have received considerable attention because they reportedly disrupt coordination and feeding of plant-sucking insects such as aphids and whiteflies and are effective against insects that have developed resistance to other insecticides, while having low acute toxicity to bees (Maienfisch, 2012). Studies on locusts have shown that PM specifically affects chordotonal neurons (CHNs) (Ausborn et al., 2005)—serially arranged stretch receptors that control body movements in insects and crustaceans (Field and Matheson, 1998; Kavlie and Albert, 2013) and allow *Drosophila* to also sense gravity and to hear (Kamikouchi et al., 2009; Yorozu et al., 2009; Sun et al., 2009). By analyzing insecticidal effects of PM and PFQ on *Drosophila* behavior and cell function, we have now identified TRP channels as their target proteins.

RESULTS

To test for insecticide effects, we kept wild-type flies for 2 hr on 1% sugar water containing 0.5% DMSO and PM or PFQ at concentrations of 200 μM. PM or PFQ rendered the flies uncoordinated and inactive, making them stay sedentary at the bottom of their vial. We quantified this behavior with a simple climbing assay (Sun et al., 2009), in which the percentage of flies is scored that climb up in darkness into the upper half of a vertical vial (Figure 1A). Control flies kept on sugar water alone or on sugar water plus DMSO displayed normal anti-gravitaxis behavior: within 30 s after being tapped down to the bottom, ca. 70% of the flies climbed up, against the Earth's gravitational field (Figure 1B, left and right panels). This anti-gravitaxis was abolished by PM or PFQ, resulting in climbing scores of consistently less than 1% (Figure 1B, left and right panels, and Movie S1). Gravitaxis defects reportedly also characterize *Drosophila nanchung*^{36a} (*nan*^{36a}) and *inactive*^{1} (*iav*^{1}) null mutants, whose CHNs are functionally impaired (Kim et al., 2003; Gong et al., 2004). Consistent with previous observations (Sun et al., 2009), we found that some residual gravitaxis persists in these mutants (Figure 1B, middle and right panels), presumably because gravity sensing is partly taken over by other mechanosensory cells when CHNs are impaired permanently (Kamikouchi et al., 2009). Neither PM nor PFQ affected this residual gravitaxis (Figure 1B, middle

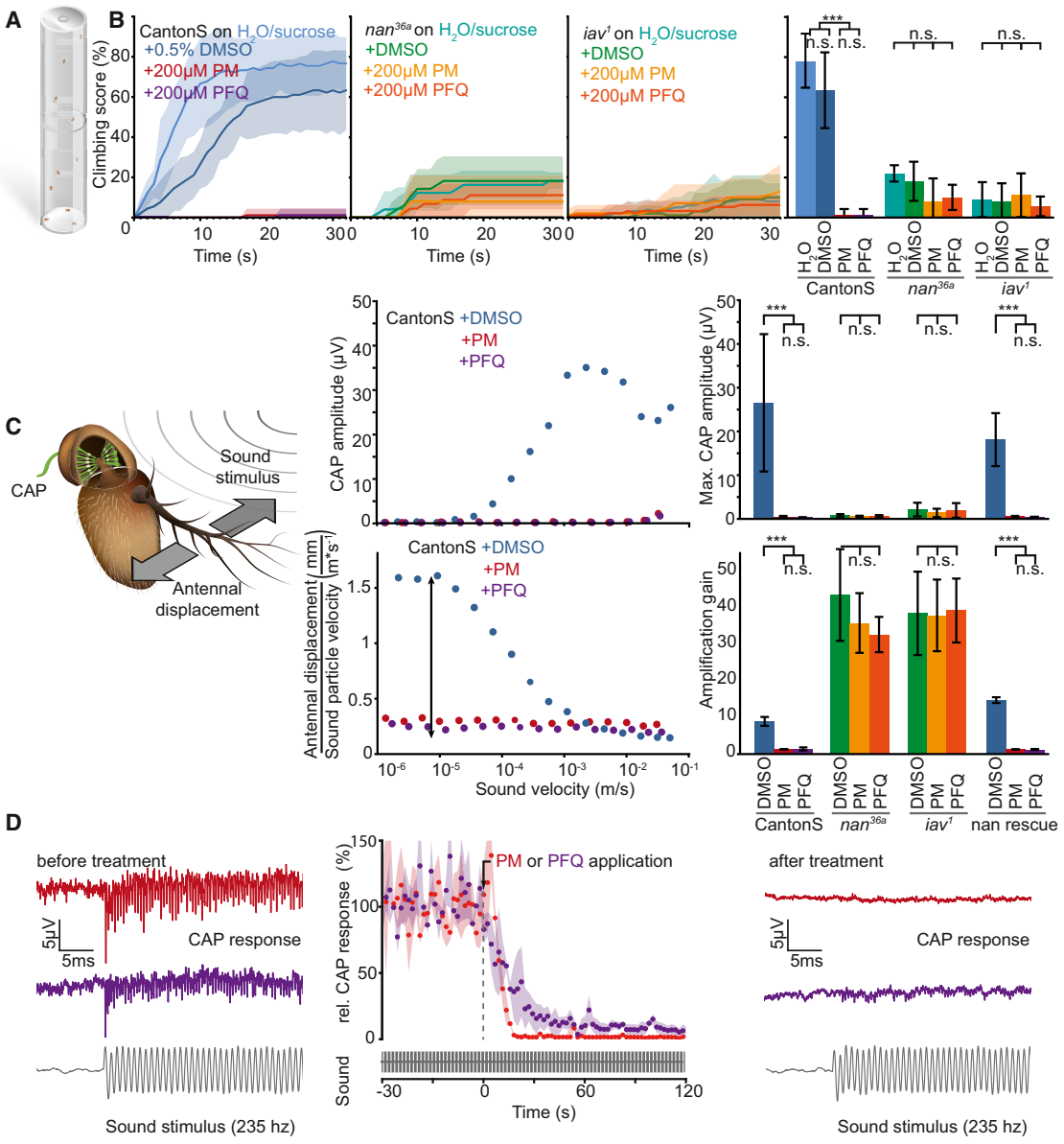

Figure 1. Insecticides Affect *Drosophila* Behavior through CHNs

(A) Climbing assay, in which the percentage of flies that climb in darkness into the upper half of their vial is scored. (B) Left: climbing scores of wild-type flies fed with and without PM or PFQ, determined at 2 s intervals, after the animals had been tapped down (N = 10 flies per vial, n = 10 repetitions each). Lines: means; colored areas: ±1 SD. Middle and right: corresponding climbing scores for nan^{36a} and iav^{1} mutants with and without PM or PFQ. (C) Left: measuring sound-induced antennal displacements and compound action potentials (CAPs) of antennal CHNs (green). Middle: CAP amplitudes (top) and corresponding mechanical susceptibility of the antenna (bottom) as functions of the sound particle velocity (example data from one animal each). In the control, CAP amplitudes reach 35 µV (top), and motile CHN responses amplify the antenna's mechanical susceptibility to faint sounds with a gain of ca. 10 (arrow). Right: maximum CAP amplitudes (top) and amplification gains (bottom) in wild-type, nan^{36a}, and iav^{1} mutants and *nan* rescue flies with and without PM or PFQ (means ± 1 SD, N = 6 flies each). ***p < 0.005, Mann-Whitney U tests with Benjamini-Hochberg correction). (D) Time course of the silencing of the tone-evoked CAPs of antennal CHNs (means ± SD data from 3 wild-type flies each) during bath application of PM and PFQ. 100% corresponds to the mean amplitude before treatment.

and right panels, see also Movie S1 and Figure S1B), documenting that nan^{36} and iav^{1} mutants show behavioral resistance to the two insecticides.

Gravity sensing and hearing in *Drosophila* are mediated by some 500 CHNs in the second segment of the fly's antenna (Kamikouchi et al., 2009; Sun et al., 2009). To test whether PM and PFQ affect CHNs, we exposed the flies to pure tones and monitored the resulting antennal displacements and associated compound action potentials (CAPs) of the antennal CHNs (Figure 1C). In wild-type flies treated with sugar water alone or sugar water plus DMSO, sound particle velocities exceeding 0.1 mm/s evoked robust CAP responses (Figure 1C, top). Sound-induced

antennal displacements exhibited the characteristic nonlinear intensity scaling that, arising from motile responses of CHNs (Göpfert et al., 2006; Nadrowski et al., 2008), mechanically amplified small antennal displacements with a gain of ten (Figure 1C, bottom). PM and PFQ abolished both these electrical and motile CHN responses, reducing the mechanical amplification gain to one (Figures 1C and 1D). Electrical CHN responses are reportedly also lost in nan^{36a} and iav^{1} mutants (Kim et al., 2003; Gong et al., 2004), yet their CHNs are still motile (Göpfert et al., 2006), providing mechanical hyper-amplification with gains of around 50 (Figure 1C, right). Unlike in wild-type flies, mechanical amplification in nan^{36a} and iav^{1} mutants was resistant to PM and PFQ (Figure 1C, see also Figure S1B). This resistance broke when we expressed a UAS-*nan* rescue construct containing an upstream activating sequence (UAS) in the nan^{36a} mutant background via the *nan* promoter fusion construct F-GAL4 (= *nan*-GAL4) (Liu et al., 2007) (Figure 1C), which also rescued Iav localization in the CHNs (Figure S2A).

nan and *iav* both encode TRP vanilloid (TRPV) subfamily members that seem conserved across insect species (Matsuura et al., 2009). Nan and Iav co-localize and presumably heteromerize in the mechanosensory cilia of CHNs, where the two proteins are abolished together in both nan^{36a} and iav^{1} nulls (Gong et al., 2004; see also Figure S2A). To test whether Nan and Iav also co-occur in cells other than CHNs, we generated flies co-expressing the promoter fusion constructs *nan*-GAL4 and *iav*-lexA, in which the *nan* and *iav* promoters are fused to the transcriptional activators GAL4 and LexA, respectively (Liu et al., 2007; Shearin et al., 2013). Driving fluorescent reporters via these constructs indicated that Nan and Iav exclusively co-occur in CHNs. Judging from the promoter fusions, *iav* seems solely expressed by CHNs, including the antennal ones and the five CHNs of the larval abdominal lateral pentascolopidial organ (lch5) (Figure 2A, Figures S2B–S2E). *nan*, by contrast, was expressed more broadly, including most CHNs as well as some multidendritic neurons (Figure 2A) and hygroreceptors in the third segment of the fly's antenna that reportedly require Nan (Liu et al., 2007) (Figure S2E). When we used *nan*-GAL4 to drive expression of the calcium sensor GCaMP6m (Chen et al., 2013), we found that bath application of PM or PFQ induces strong calcium signals in CHNs that co-express *nan* and *iav*, but not in multidendritic neurons that only express *nan* (Figure 2B). PM and PFQ also evoked strong and sustained calcium signals in antennal CHNs (Figure 2C), corroborating previous reports that PM electrically silences cells through overstimulation (Ausborn et al., 2005). For antennal CHNs, this silencing occurred gradually within about 1 min after bath application of PM or PFQ (Figure 1D), suggesting that the increased calcium levels (Figure 2C) functionally deteriorate the CHNs. No insecticide-evoked calcium responses were seen in the fly's brain (Figures 2C–2E), and calcium responses were also absent from the CHNs of nan^{36a} and iav^{1} mutants (Figures 2D–2F) as well as from muscles (Figure S2F) and the hygroreceptors in the third antennal segment that express only *nan* (Figures 2G and 2H). Misexpressing *iav* by driving a UAS-*iav* construct (Kwon et al., 2010) with the pan-neuronal driver *elav*-GAL4 conferred insecticide-evoked calcium responses to these latter hygroreceptors but not to central neurons in the brain (Figures 2G and 2H). *iav* expression thus renders *nan*-positive cells, but not *nan*-negative ones responsive to the insecticides, providing in vivo evidence that cellular insecticide actions require both Iav and Nan.

To test whether insect TRPVs can confer cellular insecticide responses in vitro, we transiently transduced hamster ovary CHO-K1 cells with adenoviruses expressing *Drosophila* Nan or Iav (Figure S3A). Because PFQ was found to deacetylate spontaneously in aqueous solution (Figure S3B), we also tested deacetylated PFQ (= dPFQ) (Figure 3; Figure S3B). Using fluo-4 as a calcium indicator (Gee et al., 2000), we found that PM, PFQ, and dPFQ evoke calcium responses in cells co-expressing Nan and Iav but not in cells expressing Nan or Iav alone (Figure 3A). Dose-response curves yielded half-maximal effective (E_{50}) concentrations of 0.1 and 0.12 μM for PM and dPFQ, respectively (Figure 3G). Compared to dPFQ, PFQ was about 100-fold less potent (E_{50} of 10.5 μM, Figure S3C), suggesting that PFQ is a prodrug that is activated through deacetylation. dPFQ evoked faster calcium responses of *Drosophila* CHNs than did PFQ (Figure S3D), providing in vivo support for such PFQ activation. Using dPFQ, maximum calcium responses were obtained when the CHO cells were co-transduced with Nan and Iav adenoviral particles at a ratio of 1:1 (Figure 3B, left). Western blotting confirmed that this co-transduction leads to approximately equal cellular Nan and Iav protein levels (Figure 3B, right), suggesting that the insecticides evoke cellular calcium signals by activating Nan-Iav complexes with a Nan:Iav stoichiometry of 1:1. To test whether Nan and Iav assemble into Nan-Iav complexes, we fused the two proteins with two different epitope tags, co-expressed them in CHO cells, and found that Nan co-immunoprecipitates with Iav protein (Figure 3C). To further test for Nan-Iav complex formation, we tagged Iav and Nan with AcGFP and mCherry moieties, respectively, and co-expressed them in CHO cells. Iav-AcGFP excitation elicited Nan-mCherry emission, documenting Förster resonance energy transfer (FRET) between the AcGFP/mCherry pair (Figure S3E). Together, these experiments document in vitro complex formation for Nan and Iav, corroborating in vivo indications that these two TRPVs form heteromers (Gong et al., 2004; Delmas and Coste, 2013).

Confocal microscopy on CHO cells revealed that the bulk of Nan and Iav proteins localizes to intracellular compartments, regardless whether they were expressed alone or together (Figure S3F). In line with previous observations (Cuajungco et al., 2006), heterologously expressed mouse TRPV4 was also found mainly inside cells (Figure S3F). Analogous to mammalian TRPVs, the two insect TRPVs thus seem to require specific stimuli and/or co-factors to facilitate their surface translocation (Venkatachalam and Montell, 2007). Notwithstanding the predominantly intracellular localization of heterologously expressed Nan and Iav, the insecticide-evoked calcium signals were found to reflect calcium entry into the CHO cells rather than internal calcium mobilization. First, the responses were abolished by the removal of calcium from the external medium, documenting that they require extracellular calcium (Figure 3D). Second, the calcium responses were blocked by ruthenium red (Figure 3E), a cell-impermeable pan-inhibitor of TRPs (Vriens et al., 2009). Third, although the low surface expression of Nan-Iav hampered the detection of insecticide-evoked currents by patch clamp,

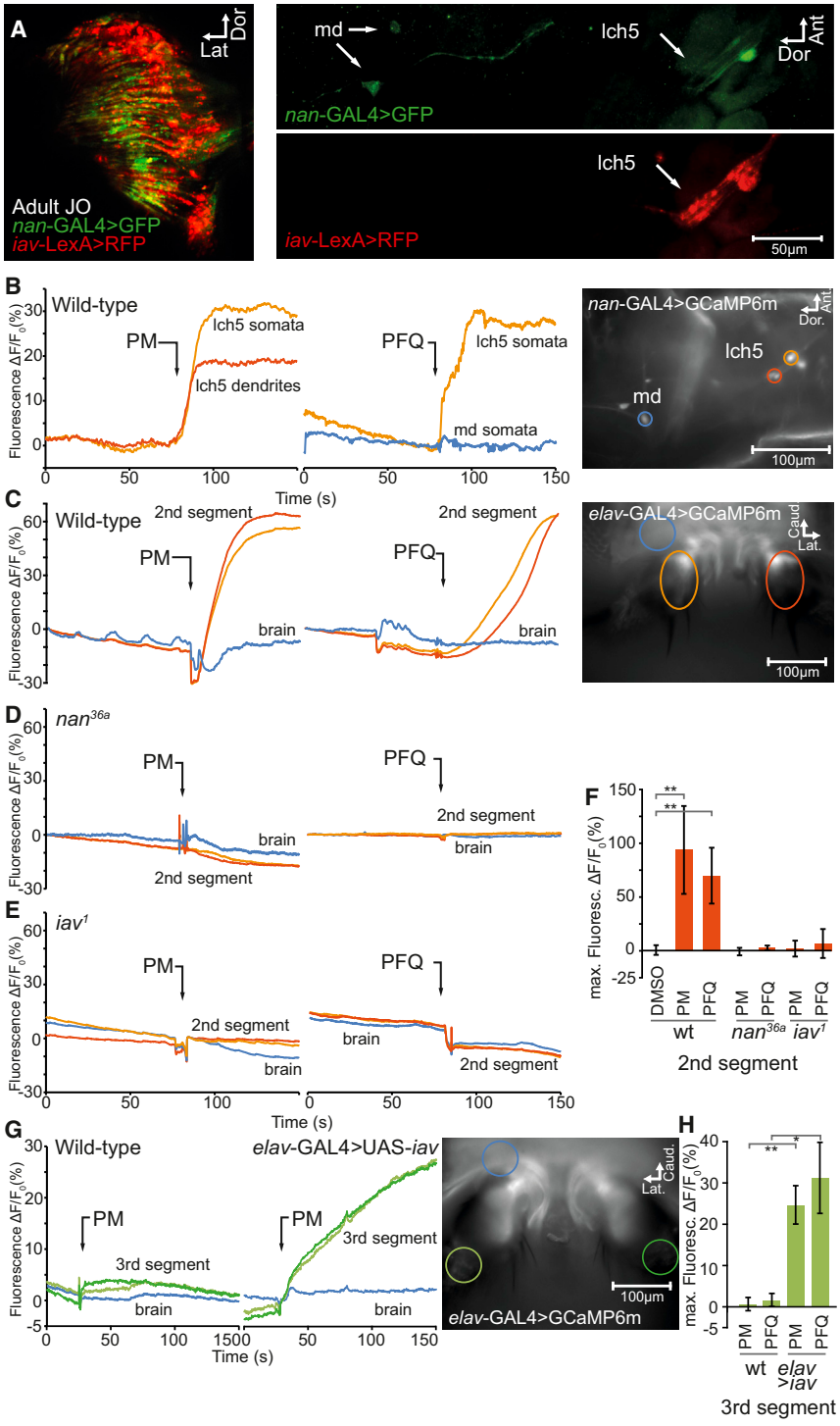

Figure 2. Insecticides Affect CHNs

(A) CHNs in the adult antenna (left) and the larval lch5 (right) co-express *nan* and *iav*, whereas some multidendritic (md) neurons express *nan* (top right) but not *iav* (bottom right). (B) Insecticide-evoked calcium responses in the larval peripheral nervous system revealed by driving GCaMP6m via *nan*-GAL4. Right: regions of interest. Left: corresponding calcium signals evoked by bath application of 50 µM PM (left) or PFQ (right) in the dendrites and somata of lch5 neurons (left), and their absence in md neurons that only express *nan* (right) (example traces from one animal each). (C–F) Insecticide-evoked calcium responses of CHNs in the second antennal segment and brain neurons revealed by expressing GCaMP6m via the pan-neural driver *elav*-GAL4. Right: regions of interest. Left: example traces of PM- and PFQ-evoked calcium signals in wild-type flies (C) and nan^{36a} (D) and iav^{1} (E) mutants. (F) Maximum calcium signals observed upon compound administration (N = 6 each, means ± SD). **p < 0.01, U tests with Benjamini-Hochberg correction. Antennal CHNs but not central brain neurons show insecticide-evoked calcium increases in wild-type flies that are lost in nan^{36a} and iav^{1} mutants (D–F). The slight movement artifacts at the beginning of the responses in (B)–(E) are caused by the insecticide injection. (G) Insecticide-evoked calcium responses of hygroreceptors in the third segment of the antenna brain neurons with and without pan-neural misexpression of *iav* (example traces from one animal each). (H) Respective maximum calcium response amplitudes. N = 6 each, means ± SD). **p < 0.01, U tests with Benjamini-Hochberg correction. Iav selectively confers calcium responses to the hygroreceptors, which also express *nan*.

a fluorescent voltage indicator whose translocation across plasma membrane depends on the membrane depolarization (Zheng et al., 2004) reported insecticide-induced changes of the cell membrane potential (Figure 3F). These potential changes resembled the calcium signals with respect to their time course (Figure 3F, left) and their dose dependence (Figure 3F, right). Like the calcium signals, the potential changes were abolished by the omission of external calcium, indicating that Nan-Iav complexes form calcium-conducting ion channels (Figure S3G). By activating a relatively low number of Nan-Iav complexes that are exposed to the surface, the insecticides thus promote calcium entry into cells.

To gain insight into the target selectivity of the insecticides, we also transduced CHO-K1 cells with the mouse TRPV channel TRPV4. To allow for comparison with cells that co-express Nan and Iav, we equalized TRPV4 protein levels with those of Nan and Iav via western blotting (Figure S3H). The TRPV4 agonist GSK1016790A (Thorneloe et al., 2008) activated TRPV4 but not Nan-Iav (Figure 3G). Conversely, PM activated Nan-Iav but not TRPV4 (Figure 3G). PFQ failed to activate TRPV4 at concentrations of up to 90 µM (Figure S3I), whereas dPFQ activated both Nan-Iav and TRPV4, though its potency for Nan-Iav was ca. 100-fold higher than for TRPV4 (E_{50}s of 0.12 and 10.5 µM, respectively; Figure 3G). Besides GSK1016790A, we tested several agonists of mammalian

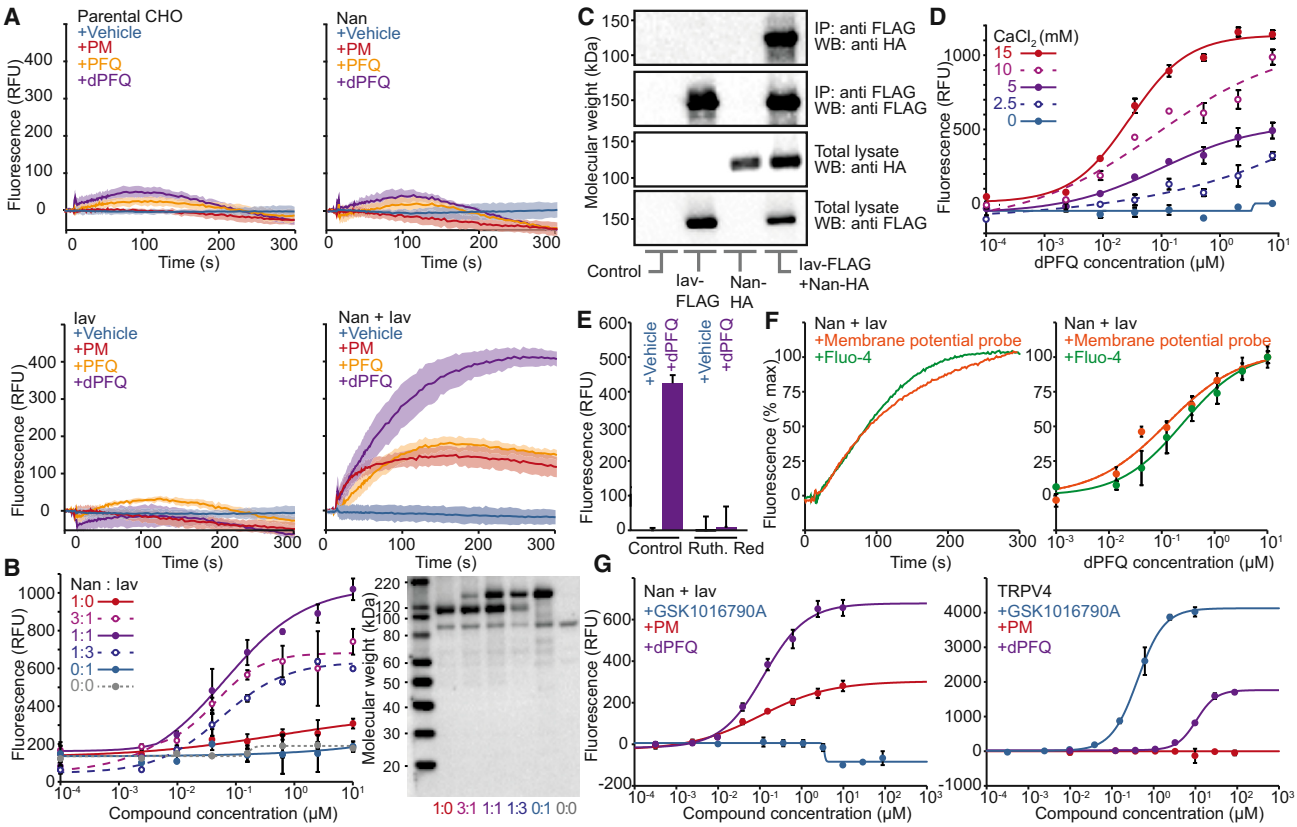

Figure 3. Insecticides Activate Nan-Iav Heteromers

(A) Insecticides (20 μM) evoke calcium signals (relative fluorescence units [RFU]) in CHO cells co-expressing Nan and Iav but not parental cells or cells expressing Nan or Iav alone (averages [lines] ± 1 SD [areas] of 4 repetitions). (B) Dose-response relationships of dPFQ-evoked calcium signals and fitted Hill equations for co-transduction with different Nan:Iav adenoviral particle ratios (left, n = 4 each). Right: respective western blot with an antibody against a common AcGFP moiety of Nan and Iav. (C) Nan and Iav co-immunoprecipitate. CHO cells were transduced with FLAG-tagged Iav and/or HA-tagged Nan, and FLAG-Iav was immunoprecipitated (IP) with an anti-FLAG antibody. (D) Dose-response relationships of dPFQ-evoked calcium responses of cells co-expressing Nan and Iav at different external calcium concentrations (n = 4). (E) Maximum calcium responses evoked by 20 μM dPFQ or 0.2% DMSO in cells transduced with Nan and Iav, with (controls) and without ruthenium red (n = 4). (F) dPFQ-evoked calcium and membrane potential signals as functions of time (left) and the dPFQ concentration (right) (n = 4). (G) Dose response of CHO cells transduced with Nan and Iav (left) or mouse TRPV4 (right) for PM, dPFQ, and GSK1016790A (n = 4).

TRPVs (Vriens et al., 2009), but none of them activated the co-expressed Nan and Iav proteins (Figure S3J). Contrasting with previous observations (Kim et al., 2003; Gong et al., 2004), hypotonic stimuli failed to activate CHO cells expressing Nan or Iav alone or Nan and Iav together, whereas TRPV4 conferred hypotonically evoked responses to CHO cells (Figure S3K), consistent with previous reports (Liedtke et al., 2000; Strotmann et al., 2000; Wissenbach et al., 2000).

DISCUSSION

PM and PFQ are the first specific agonists of insect TRPs and the first insecticides that are shown to target TRPs. By activating Nan-Iav TRPV channel complexes, both insecticides impair insect coordination by affecting CHNs. This cell-type-specific insecticidal action is supported by the behavioral insecticide resistance of *Drosophila* mutants with impaired CHNs (Figure 1A), as well as by the absence of insecticide-evoked responses from other sensory neurons (Ausborn et al., 2005), the CNS (Figures 1C–1E), and muscles (Figure S2F). The cell-type selectivity is shown to reflect the selective co-occurrence of Nan and Iav in CHNs, where they have been proposed to mediate mechanosensory stimulus transduction (Lehnert et al., 2013). The absence of insecticide-evoked responses from muscles is consistent with a recently reported role of Iav, but not Nan, at the neuromuscular junction (Wong et al., 2014). Our inability to reproduce the reported hypotonic activation of Nan and Iav in CHO cells (Kim et al., 2003; Gong et al., 2004) raises the need to revisit the activation mechanisms and roles of these channels in mechanotransduction and CHN function.

Nan and Iav together are shown to be required and sufficient to confer cellular insecticide responses in vivo (Figures 2D–2H) and in vitro (Figure 3), promoting cellular calcium entry that seems to electrically silence the CHNs (Figure 1D). Nan and Iav are further shown to assemble into functional Nan-Iav complexes (Figures 3C and S3E), corroborating previous immunohistological in vivo indications for their heteromerization (Gong et al., 2004). Activating Nan-Iav complexes, but not their single

subunits, PM and PFQ are remarkable in that they selectively stimulate two interacting TRPs, making them useful tools to specifically probe the permeation properties of a heteromeric TRP complex and its activation mechanisms. Judging from our data, PM and PFQ seem to activate Nan-Iav directly, yet further studies will be required to test the directness of this activation and to assess whether the insecticides physically bind to Nan-Iav.

The apparent cell specificity conferred by their TRP targets distinguishes PM and PFQ from other commercial insecticides that act rather broadly on insect neurons or muscles. The use of a different molecular target may explain in some cases why insects resistant to other insecticides are still susceptible to PM and PFQ (Maienfisch, 2012). Because Nan and Iav seem conserved across insects, one would expect the two insecticides to broadly act on insect species. Indeed, although PM and PFQ are primarily used to control plant-sucking hemipteran insects, they reportedly also affect thysanopteran (Maienfisch, 2012), orthopteran (Ausborn et al., 2005; Möckel et al., 2011), and coleopteran (Tait et al., 2011; Chang and Snyder, 2008; Cole et al., 2010) insects and have nonlethal effects on honey bees (Maienfisch, 2012). The different strengths of the effects might reflect sequence variations of Nan-Iav and/or differences in the importance of CHNs for insect survival: PM and PFQ are described as feeding blockers that disrupt feeding in plant-sucking insects (Maienfisch, 2012). Upon treatment, these insects starve because they can no longer penetrate plants with their mouthparts (Maienfisch, 2012). This ultimately lethal effect seems to contrast with the persistent viability of *Drosophila* and locusts (Ausborn et al., 2005). The dispensability of CHNs for *Drosophila* survival is illustrated by the fact that *nan* and *iav* mutants are viable and develop to adults without functional CHNs (Kim et al., 2003; Gong et al., 2004). Possibly, movement control by CHNs is particularly crucial for inserting the mouthparts into plant tissues, making plant-sucking insects particularly vulnerable to PM and PFQ. Differences in insect feeding styles might also explain the reportedly low acute toxicity of the two insecticides for honey bees. In addition, we anticipate that targeting TRPs with insecticides might help to reduce potential side effects on pollinators: insect TRPP, for example, which is implicated in *Drosophila* male fertility (Gao et al., 2003), is absent in lepidopterans and hymenopterans, including bees (Matsuura et al., 2009).

EXPERIMENTAL PROCEDURES

Animals

Flies were maintained in accordance with German Federal regulations (license Gen.Az 501.40611/0166/501). Specific details regarding the strains used in the experiments can be found in the Supplemental Experimental Procedures.

Behavioral Analyses

Tube-climbing assays were carried out under infrared illumination essentially following established protocols (Sun et al., 2009).

In Vivo Cell Responses

The methods to access sound-evoked electrical and motile CHN responses have previously been described (Albert et al., 2006). Live imaging of intracellular calcium responses was performed following established protocols (Kamikouchi et al., 2010; Parton et al., 2010).

In Vitro Cell Responses

CHO-K1 cells were transduced with adenoviruses expressing *Drosophila Nan* or *Iav* either alone or in combination, or mouse *TRPV4*, as fusion proteins containing AcGFP and FLAG tags at carboxyl termini (for details, see Supplemental Experimental Procedures). Cells were seeded on 96-well plates and their insecticide responses were assessed using a Fluorometric Imaging Plate Reader (Marshall et al., 2013).

Statistical Analyses

Statistical comparison of means was performed using two-tailed Mann-Whitney U tests and significance was concluded when $p < 0.05$. Unless otherwise stated, data are presented as mean ± 1 SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.04.001.

AUTHOR CONTRIBUTIONS

A.N., C.S., and R. Kandasamy contributed equally to this work. A.N. designed and coordinated in vitro experiments, C.S. performed and analyzed most in vivo experiments and devised figures, and R. Kandasamy conducted and analyzed in vitro experiments. R. Katana, B.W., P.J., and V.L.S. studied mechanically evoked CHN responses, M.A. analyzed expression patterns, N.B.R., L.S., and J.A.D. helped with heterologous expression, and F.-J.B. designed calcium selectivity experiments. V.L.S. and M.C.G. initiated and coordinated the work, and M.C.G. wrote the manuscript with A.N., C.S., V.L.S., and N.B.R.

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