

Drosophila epidermal cells are intrinsically mechanosensitive and modulate nociceptive behavioral outputs

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eLife Assessment

This is **important** work and provides a significant advance in our understanding of mechanosensation in the epidermis. The evidence presented is **convincing** and, barring a few minor weaknesses, strongly implicates activation of epidermal cells and store-operated calcium entry in the activation of nociceptive neurons innervating that tissue. This work will be of broad interest to neurobiologists, epithelial cell biologists, and mechanobiologists.

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Abstract

Somatosensory neurons (SSNs) that detect and transduce mechanical, thermal, and chemical stimuli densely innervate an animal's skin. However, although epidermal cells provide the first point of contact for sensory stimuli, our understanding of roles that epidermal cells play in SSN function, particularly nociception, remains limited. Here, we show that stimulating *Drosophila* epidermal cells elicits activation of SSNs including nociceptors and triggers a variety of behavior outputs, including avoidance and escape. Further, we find that epidermal cells are intrinsically mechanosensitive and that epidermal mechanically evoked calcium responses require the store-operated calcium channel Orai. Epidermal cell stimulation augments larval responses to acute nociceptive stimuli and promotes prolonged hypersensitivity to subsequent mechanical stimuli. Hence, epidermal cells are key determinants of nociceptive sensitivity and sensitization, acting as primary sensors of noxious stimuli that tune nociceptor output and drive protective behaviors.

Introduction

The ability to detect tissue-damaging noxious stimuli and mount an escape response is essential for survival. Likewise, prolonged hypersensitivity following injury is an important form of plasticity that protects an animal from further damage. In *Drosophila*, a single class of identified somatosensory neurons (SSNs), class IV dendrite arborization (C4da) neurons, are necessary and sufficient for nociception; inactivating C4da neurons renders larvae insensitive to noxious stimuli whereas activating these neurons drives nocifensive behavior responses (Hwang *et al.*, 2007 [↗](#); Hu *et al.*, 2017 [↗](#); Burgos *et al.*, 2018 [↗](#)). A variety of agents that cause tissue damage including UV irradiation and chemical toxins induce long-lasting allodynia and hyperalgesia (Babcock *et al.*, 2009 [↗](#); Boiko *et al.*, 2017 [↗](#)), but this damage-induced hypersensitivity develops on a timescale of hours. *Drosophila* also display acute hypersensitivity to noxious mechanical stimuli (Hu *et al.*, 2017 [↗](#)). However, the cellular and molecular mechanisms underlying mechanical pain hypersensitivity remain enigmatic.

Recent studies demonstrate that epidermal cells work in concert with SSNs to transduce noxious and innocuous physical stimuli. For example, epidermal Merkel cells are mechanosensory cells that signal to sensory neurons to mediate touch transduction (Maksimovic *et al.*, 2014 [↗](#); Hoffman *et al.*, 2018 [↗](#)). Similarly, keratinocytes are directly activated by noxious thermal and mechanical stimuli and release molecules that modulate nociceptor functions (Chung *et al.*, 2004 [↗](#); Koizumi *et al.*, 2004 [↗](#); Moqrich *et al.*, 2005 [↗](#); Mandadi *et al.*, 2009 [↗](#); Liu *et al.*, 2019 [↗](#); Sadler *et al.*, 2020 [↗](#)). Furthermore, epidermal cells in invertebrates and vertebrates ensheath nociceptors in mesaxon-like structures (Cauna, 1973 [↗](#); Chalfie and Sulston, 1981 [↗](#); Han *et al.*, 2012 [↗](#); Kim *et al.*, 2012a [↗](#); O'Brien *et al.*, 2012 [↗](#); Jiang *et al.*, 2019 [↗](#)), and these sheaths may serve as sites of epidermis-nociceptor signaling (Yin *et al.*, 2021 [↗](#)). Indeed, epidermal ensheathment is required for normal responses to noxious mechanical stimuli in *Drosophila* (Jiang *et al.*, 2019 [↗](#)). However, whether epidermal cells are directly activated by noxious stimuli and modulate C4da neuronal activity has not been studied.

Here, we examined the capacity of *Drosophila* epidermal cells to drive nociceptor activation and modulate mechanical nociceptive responses. We found that stimulation of epidermal cells, but no other non-neuronal cell types in the larval body wall evokes activity in a variety of SSNs neurons and triggers nocifensive behavioral responses. Our *in vitro* and *ex vivo* calcium imaging experiments demonstrate that epidermal cells are intrinsically mechanosensitive. Using an unbiased genetic screen, we discovered a role for the store-operated calcium channel Orai, and its activator Stim in epidermal mechanotransduction and mechanical sensitization. Downstream of Stim/Orai activation, epidermal cells evoke nociceptor activation and mechanical hypersensitivity via epidermal vesicular release. Overall, we demonstrate that *Drosophila* epidermis-neuron signaling mediates both the acute detection of noxious mechanical stimuli and a form of prolonged mechanical hypersensitivity.

Results

Stimulation of epidermal cells evokes nocifensive behavior

To identify peripheral non-neuronal cell types that contribute to nociception, we conducted an optogenetic screen for light-evoked nocifensive behavior. First, as a benchmark for comparison we used the light-activated cation channel CsChrimson (Klapoetke *et al.*, 2014 [↗](#)) to optogenetically activate nociceptive C4da neurons.

Consistent with prior reports (Hwang *et al.*, 2007 [↗](#); Hu *et al.*, 2017 [↗](#)), C4da activation triggered nocifensive behaviors including c-bending and rolling in 100% of larvae (**Fig. 1A** [↗](#), Fig. 1S1A, Movie S1). Next, we selectively expressed CsChrimson using GAL4 drivers in combination with *elav-GAL80*, which effectively silences GAL4 expression in larval sensory neurons (Fig. 1S2), to target the six principle non-neuronal cell types within the larval body wall: epidermis, trachea, muscle, hemocytes, oenocytes, and glia (Fig. 1S3, Key Resources Table). We then monitored light-evoked behavioral outputs associated with stimulation of each cell type. We found that optogenetic stimulation of epidermal cells, like C4da neurons, elicited nocifensive c-bending and/or rolling behaviors in 73% of larvae (**Fig. 1A** [↗](#), 1S1B), without significantly altering nociceptor morphogenesis (Fig. 1S4). In contrast, stimulation of other body wall cell types elicited a variety of non-nociceptive behavior outputs: for example, muscle stimulation triggered hunching behavior followed by prolonged freezing, whereas glia stimulation reproducibly induced only hunching behavior (Fig. 1S1C-I) (Zimmermann *et al.*, 2009 [↗](#)). Thus, epidermal cells are the only non-neuronal body wall cell type that triggers robust nocifensive behavioral responses.

To further validate the selective ability of body wall epidermal cells to drive nocifensive behaviors, we examined eight other epidermal drivers in addition to *R38F11-GAL4*, which displays no expression in sensory neurons and limited non-epidermal cell expression overall (Fig. 1S5). We found that optogenetic stimulation evoked nocifensive behaviors with each of the eight epidermal driver lines we tested: seven of the lines displayed rolling behavior while all eight displayed c-bending (**Fig. 1B** [↗](#), Fig. 1S6). Although the previously described pan-epidermal *A58-GAL4* driver (Galko and Krasnow, 2004 [↗](#)) drove robust nocifensive rolling responses (**Fig. 1B** [↗](#), 1S6), *A58-GAL4* is expressed broadly in the larval CNS (Fig. 1S7) and stochastically expressed in sensory neurons (Jiang *et al.* 2014 [↗](#)). In contrast, the remaining seven drivers including *R38F11-GAL4* exhibited limited expression aside from epidermal cells, with no detectable expression in nociceptors, other larval SSNs, or peripheral glia, and highly restricted or undetectable expression in the CNS (Fig. 1S7). Further underscoring the connection between epidermal stimulation and nocifensive responses, the nocifensive behavioral response with these epidermal drivers correlated with the proportion of epidermal expression (**Fig. 1B** [↗](#)).

We next used thermogenetic stimulation with the warmth-activated TRP channel dTRPA1 (Hamada *et al.*, 2008 [↗](#)) as an independent method of probing nociceptive responses triggered by epidermal cell activation. On its own, the thermal stimulus (35° C) rarely induced rolling behavior in control larvae bearing *UAS-TRPA1* alone. In contrast, we found that >75% of larvae expressing TRPA1 in all nociceptors exhibited rolling behavior in response to a thermal stimulus (**Fig. 1C** [↗](#)). Likewise, thermogenetic activation of epidermal cells induced robust rolling responses in >75% of larvae, and addition of GAL80 transgenes (*tsh-GAL80 elav-GAL80*) that silenced the sparse *R38F11-GAL4* VNC expression (Fig. 1S5) had no effect on the rolling frequency (**Fig. 1C** [↗](#), Fig. 1S3). Altogether, these results demonstrate that epidermal stimulation evokes nocifensive responses in *Drosophila*. Of note, prior studies demonstrated that sparse thermogenetic activation of nociceptors (<5 cells) yielded no significant increase in nocifensive rolling whereas activation of >10 cells was required to elicit rolling responses in a majority of larvae (Robertson *et al.*, 2013 [↗](#)). Hence, epidermal stimulation likely engages numerous C4da neurons to elicit these behavioral responses.

In addition to C4da nociceptors, the epidermis is innervated by a variety of other SSNs including mechanosensory C3da and chordotonal (Cho) neurons and proprioceptive C1da neurons. Whereas direct stimulation of C4da nociceptors principally elicited nocifensive behavioral outputs, epidermal stimulation elicited an array of behaviors in addition to nocifensive responses, including freezing and hunching (**Fig. 2A** [↗](#), **2B** [↗](#), **Fig. 2** [↗](#) video 1 and 2), behaviors associated with stimulation of C3da and Cho neurons (Zhang *et al.*, 2013 [↗](#); Turner *et al.*, 2016 [↗](#)). These data suggest that epidermal cells may broadly modulate SSN activity in *Drosophila*.

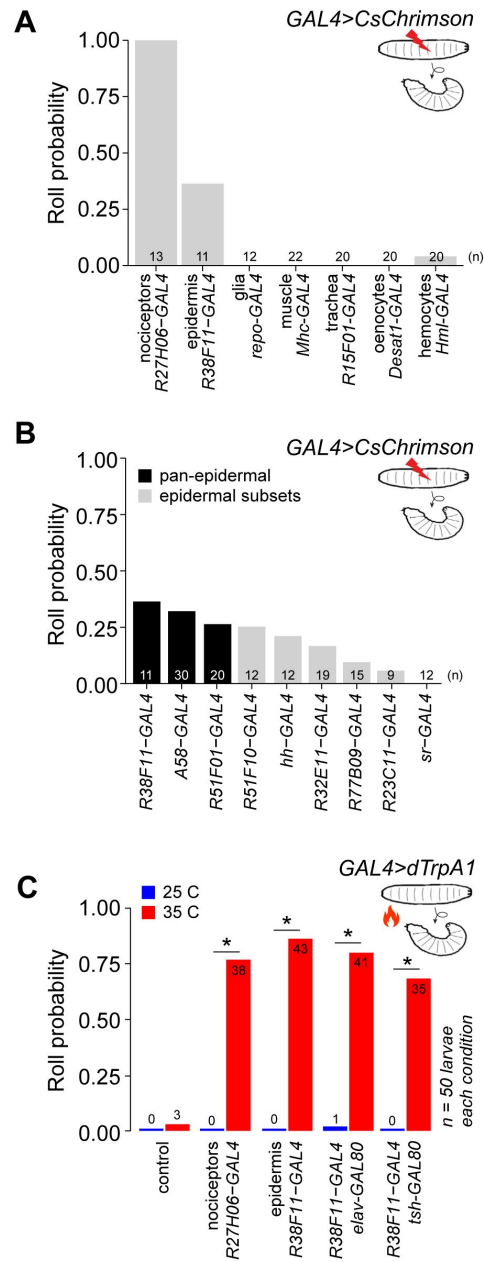


Figure 1.

Stimulation of epidermal cells elicits nociceptive behaviors.

(A) Fraction of larvae that exhibited optogenetic-induced rolling (roll probability) using the indicated GAL4 lines to drive *UAS-CsChrimson* expression. All experimental genotypes, except for larvae expressing *UAS-CsChrimson* in C4da neurons, included *elav-GAL80* to suppress neuronal GAL4 activity. Genotypes: *GAL4*, *UAS-CsChrimson*, *elav-GAL80/+*. (B) Roll probability of larvae following optogenetic stimulation using the indicated GAL4 lines in combination with *elav-GAL80* (or *tsh-GAL80* + *cha-GAL80* in the case of *A58-GAL4*) to drive *UAS-CsChrimson* expression in epidermal cells. All epidermal drivers except for *sr-GAL4*, which is expressed in apodemes but no other epidermal cells, elicited rolling responses. Genotypes: *GAL4*, *UAS-CsChrimson*, *elav-GAL80/+*. (C) Roll probability of larvae following thermogenetic stimulation using the indicated GAL4 line to express the warmth (35°C)-activated *UAS-TrpA1*. The number of rolling larvae (out of 50) is indicated for each group. Genotypes: *GAL4*, *UAS-TrpA1*, *GAL80* (as indicated)/+. Control, *UAS-TrpA1/+*. Sample sizes are indicated in each panel. Asterisk (*) indicates $p < 0.05$ in this and subsequent figures. Raw data for all figures is provided in Source Data File 1 and details of statistical analyses, including tests performed, p-values, and q-values are provided in Supplementary File 1.

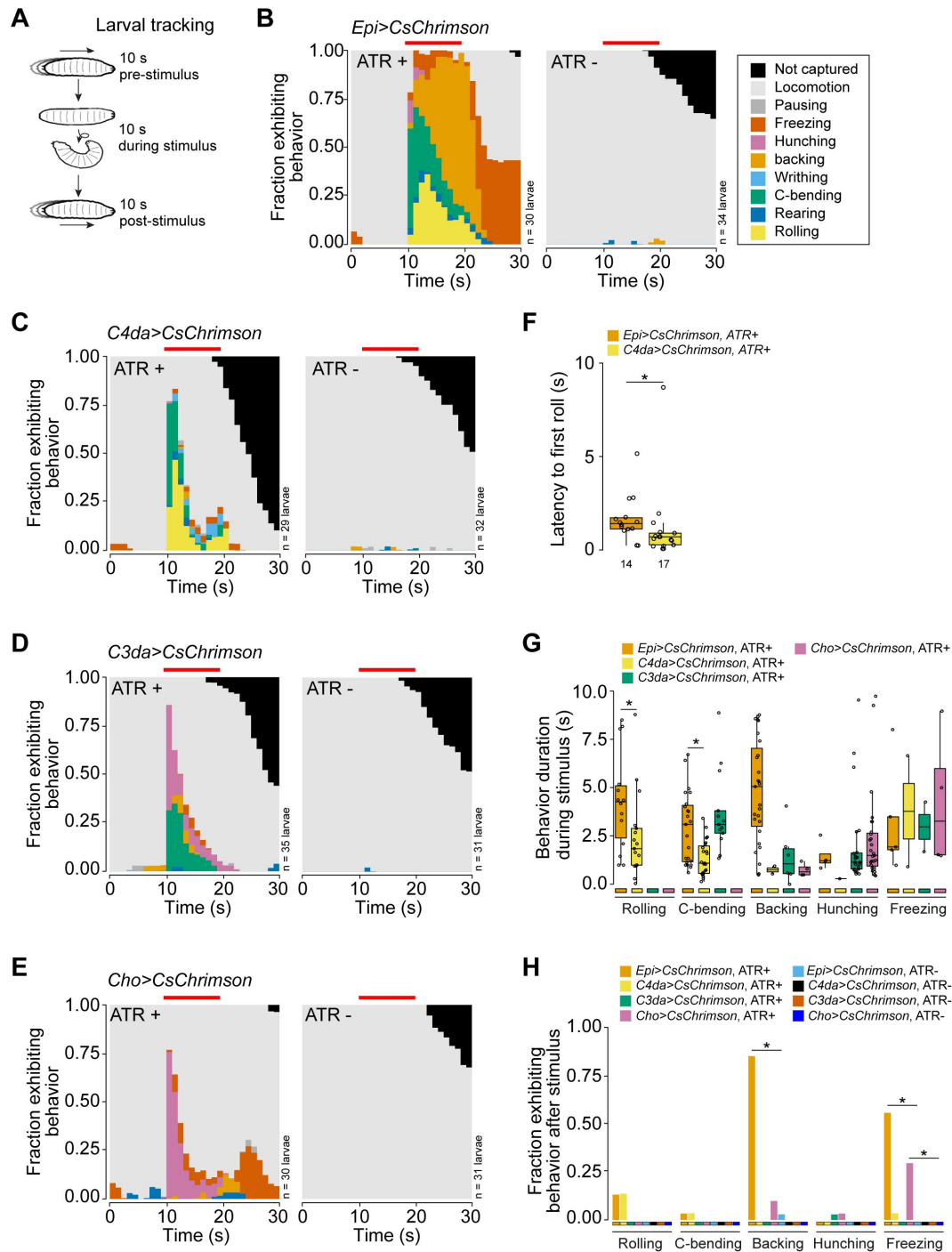


Figure 2.

Stimulation of epidermal cells evokes multimodal behavioral responses. (A) Larval behaviors were scored for 10 s before, during, and after optogenetic stimulation. (B-E) Fraction of larvae exhibiting indicated behaviors over time in one second bins expressing CsChrimson in (B) epidermal cells, (C) C4da neurons, (D) C3da neurons, and (E) Cho neurons in the presence and absence of all-trans retinal (ATR). Red line indicates the presence of light stimulation. (F) The latency to the first roll of the larvae that rolled from *Epi>Chrimson* ATR+ and *C4da>Chrimson* ATR+ treatment groups (n = 14, 17, respectively). (G) The duration of indicated behaviors of the larvae that displayed those behaviors during optogenetic stimulation. (H) The fraction of larvae that exhibited indicated behaviors following removal of the light stimulus of all larvae from panels (B-E). Genotypes: *GAL4, UAS-CsChrimson/+*.

To examine whether different epidermis-evoked behaviors were associated with activation of distinct classes of SSNs, we compared epidermis-evoked and SSN-evoked behaviors. Stimulation of C4da, C3da and Cho neurons elicited distinct behavioral motifs: only C4da neurons elicited rolling behavior; stimulation of C3da and Cho neurons together elicited hunching, C-bending, and backing; stimulation of Cho neurons alone principally elicited hunching and freezing responses (**Fig. 2A**–**2C**, 2F). In contrast, optogenetic epidermal stimulation elicited all of these behaviors, with nocifensive behaviors (c-bending, rolling) predominating initially, followed by non-nociceptive behaviors (backing, freezing) (**Fig. 2D**, **2F**, 2S1). We note that neither the behavioral motifs induced by epidermal or SSN stimulation nor the behavioral sequence induced by epidermal stimulation was recapitulated in effector-only controls (*UAS-CsChrimson ATR+*; Fig. 2S1E), demonstrating that the observed responses were driven by activation of the respective cell types.

We observed three striking differences in behavior evoked by stimulation of epidermal cells versus individual SSNs. First, although rapid, latency to rolling was significantly longer following epidermal stimulation compared to stimulation of C4da (**Fig. 2F**). Second, the duration of rolling, bending, and backing responses was significantly longer for epidermis versus SSN stimulation (**Fig. 2G**). Third, backing and freezing behaviors persisted beyond the duration of the light stimulus for epidermis but not SSN stimulation (**Fig. 2H**). In summary, we find that epidermal stimulation triggers more robust, varied and prolonged behaviors compared to responses from direct stimulation of discrete SSN subtypes.

Somatosensory neurons are activated by epidermal stimulation

We next asked whether epidermal stimulation activates larval SSNs including C4da, C3da, C1da, and Cho neurons. To test this possibility, we developed a semi-intact larval preparation in which we optogenetically stimulated epidermal cells while simultaneously monitoring calcium responses in axon terminals of SSNs (**Fig. 3A**). We found that epidermal stimulation triggered rapid and robust calcium transients in nociceptive C4da neurons, responses that were not observed in the absence of ATR or in effector-only controls (**Fig. 3B**). Epidermal stimulation likewise evoked calcium transients in mechanosensory C3da and Cho neurons, and in proprioceptive C1da neurons (**Fig. 3C**–3E, 3S1). Hence, epidermal stimulation can broadly modulate activity of larval SSNs.

We next tested the requirement for SSN synaptic transmission in epidermis-evoked behaviors. We stimulated epidermal cells with CsChrimson while blocking SSN neurotransmitter release using tetanus toxin light chain (TnT) (Sweeney *et al.*, 1995). We found that inhibiting C4da or C3da + Cho neurotransmission significantly reduced the frequency and duration of epidermal-evoked rolling and backing behaviors, respectively (**Fig. 3F**, **3G**, 3S2). These data suggest that C4da and C3da/Cho neurons act downstream of epidermal cells to drive behaviors. We note that TnT expression in C4da neurons did not completely block epidermis-evoked nocifensive behaviors, and this likely reflects both incomplete C4da neuron silencing and epidermal activation of other SSNs that promote nociceptive outputs including C3da neurons, C2da neurons, and Cho neurons (Ohyama *et al.*, 2015; Hu *et al.*, 2017; Burgos *et al.*, 2018). Further, silencing C4da or C3da/Cho neurons while stimulating epidermal cells led to an increase in the non-nocifensive behaviors hunching and freezing (**Fig. 3F**, 3G). These results, along with the observation that rolling behaviors predominate the early behavioral responses to epidermal stimulation (**Fig. 2B**), suggest that the nervous system prioritizes nocifensive behavioral outputs following epidermal stimulation. These data support a model in which epidermal cells and SSNs are functionally coupled.

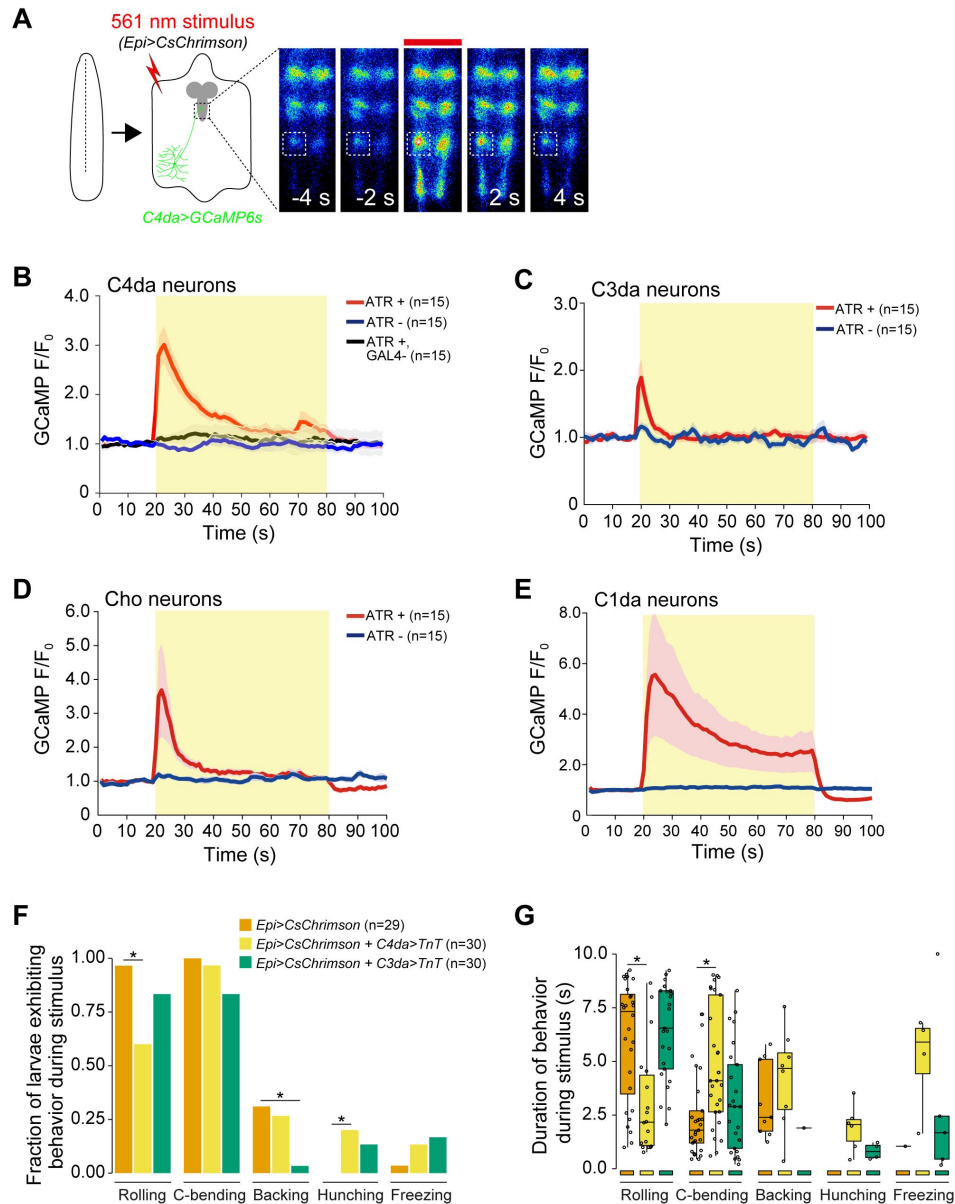


Figure 3.

(A) Optogenetic activation of CsChrimson-expressing epidermal cells in the body wall triggers calcium transients in the axon terminal of GCaMP6s-expressing nociceptive SSNs. Images show responses from one representative animal. Plots depict mean GCaMP6s fluorescence intensity of the axon terminals of (B) C4da, (C) C3da, (D) Cho, and (E) C1da neurons following optogenetic activation (light stimulus, yellow box) of epidermal cells over time. Solid lines depict mean GCaMP6s fluorescence across replicates (n=15 larval fillet preparations), shading indicates SEM, red traces are *GAL4+ ATR+*, blue traces are *GAL4+ ATR-*, black trace is *GAL4- ATR+*. (F) The fraction of larvae exhibiting indicated behaviors during optogenetic epidermal stimulation in combination with SSN silencing via Tetanus Toxin (TnT) expression. We note that although baseline rolling probability is elevated in all genetic backgrounds containing the *AOP-LexA-TnT* insertion, silencing C4da and C3da neurons significantly attenuates responses to epidermal stimulation. (G) The duration of the behavioral responses during optogenetic epidermal stimulation with neuronal TnT expression. Genotypes: (A-B) *R27H06-LexA* (C4da neurons), *AOP-GCaMP6s*, *UAS-CsChrimson/+*; *R38F11-GAL4/+* or *R27H06-LexA* (C4da neurons), *AOP-GCaMP6s*, *UAS-CsChrimson/+* (*GAL4-ATR* effector-only control); (C) *AOP-GCaMP6s*, *UAS-CsChrimson/+*; *R38F11-GAL4/NompC-LexA* (C3da neurons); (D) *UAS-GCaMP6s*, *AOP-CsChrimson*, *R61D08-GAL4* (Cho neurons)/*R38F11-LexA*; (E) *UAS-GCaMP6s*, *AOP-CsChrimson*, *R11F05-GAL4* (C1da neurons)/*R38F11-LexA*; (F-G) *R38F11-GAL4*, *UAS-CsChrimson*, *AOP-LexA-TnT/+* (*Epi>CsChrimson*); *R38F11-GAL4*, *UAS-CsChrimson*, *AOP-LexA-TnT/ppk-LexA* (*Epi>CsChrimson + C4da>TnT*); *R38F11-GAL4*, *UAS-CsChrimson*, *AOP-LexA-TnT/NompC-LexA* (*Epi>CsChrimson + C3da>TnT*).

Epidermal stimulation potentiates nociceptive neurons and behaviors

What is the physiological relevance of this functional coupling of epidermal cells and SSNs? To address this question, we compared calcium responses in C4da neurons to either simultaneous epidermal and C4da stimulation or C4da stimulation alone. Simultaneous stimulation significantly enhanced the magnitude and duration of calcium responses in C4da axons (**Fig. 4A–4D**, 4S1). Based on this prolonged calcium response, we hypothesized that simultaneous epidermis and C4da neuron stimulation would yield enhanced nocifensive behavior output. To test this, we optogenetically stimulated C4da neurons and epidermal cells individually or in combination using low intensity CsChrimson activation and monitored larval behavior responses. In this stimulation paradigm, simultaneous epidermal cell and C4da neuron stimulation resulted in rolling in 100% of larvae whereas selective stimulation of C4da neurons or epidermal cells induced rolling in only 63% or 18% of larvae, respectively (**Fig. 4E**, 4F). Furthermore, simultaneous stimulation elicited a significantly higher number of rolls among responders than stimulation of nociceptors or epidermal cells alone (26.9 rolls for C4da + Epi, 4.9 for C4da, and 5.3 for Epi stimulation; **Fig. 4G**, 4H). Likewise, simultaneous stimulation significantly reduced the latency to the first roll (**Fig. 4I**) and increased the duration of rolling behaviors (**Fig. 4J**). We next tested whether this functional coupling extends to mechanical stimuli. We simultaneously presented larvae with a noxious mechanical stimulus and a low intensity optogenetic epidermal stimulus that was insufficient to trigger rolling on its own (0% response rate, $n = 200$). This concurrent epidermal stimulation significantly increased touch-evoked nocifensive responses, yielding a 91% or 49% increase in rolling responses to 20 mN or 50 mN Von Frey stimulus, respectively (**Fig. 4K**). We next probed the kinetics of this epidermis-induced mechanical sensitization.

When *Drosophila* larvae are presented with two nociceptive mechanical stimuli in succession, they exhibit enhanced behavioral responses to the second stimulus (Hu *et al.*, 2017). We hypothesized that selective epidermal stimulation would sensitize larvae to subsequent nociceptive mechanical stimuli. To test this hypothesis, larvae expressing the warmth-activated calcium-permeable channel dTRPA1 in epidermal cells were presented with a thermal stimulus, 32° C to activate dTRPA1, followed by a 40 mN mechanical stimulus 10 s later (**Fig. 4L**). Indeed, we found that dTRPA1-mediated epidermal stimulation significantly sensitized larvae to a subsequent mechanical stimulus, increasing the roll probability more than two-fold. In contrast, dTRPA1-mediated stimulation of C4da neurons did not induce mechanical sensitization, and we confirmed this result with two independent C4da neuron drivers (**Fig. 4L**). Thus, activation of epidermal cells but not C4da nociceptors alone induces prolonged sensitization to noxious mechanical stimuli. We next assessed the duration of sensitization following transient epidermal activation. Thermogenetic epidermal stimulation yielded persistent sensitization that recovered over a timescale of minutes ($\tau = 337$ sec, **Fig. 4M**, 4N). The magnitude and duration of mechanical sensitization by thermogenetic epidermal stimulation was remarkably similar to sensitization evoked by a prior mechanical stimulus (63% roll probability in response to a second stimulus, $\tau = 334$ sec, **Fig. 4N**, 4S1B). Altogether our data support a model whereby epidermal cells are mechanosensitive cells that signal to SSNs to drive acute nocifensive behaviors and prolong mechanical sensitization.

Epidermal cells are intrinsically mechanosensitive

Prior studies have shown that vertebrate epidermal cells directly respond to mechanical stimuli (Koizumi *et al.*, 2004; Haerberle *et al.*, 2008; Tsutsumi *et al.*, 2009; Ranade *et al.*, 2014; Woo *et al.*, 2014; Moehring *et al.*, 2018). Therefore, we next assessed whether *Drosophila* epidermal cells are intrinsically mechanosensitive. We developed a protocol to acutely dissociate epidermal cells and measure the responses of individual GCaMP6s-expressing epidermal cells to mechanical stimuli (**Fig. 5A**). We found that radial stretch elicits calcium responses in epidermal cells in a dose-dependent manner. For example, a low 0.5% stretch activated 18% of cells and a subsequent

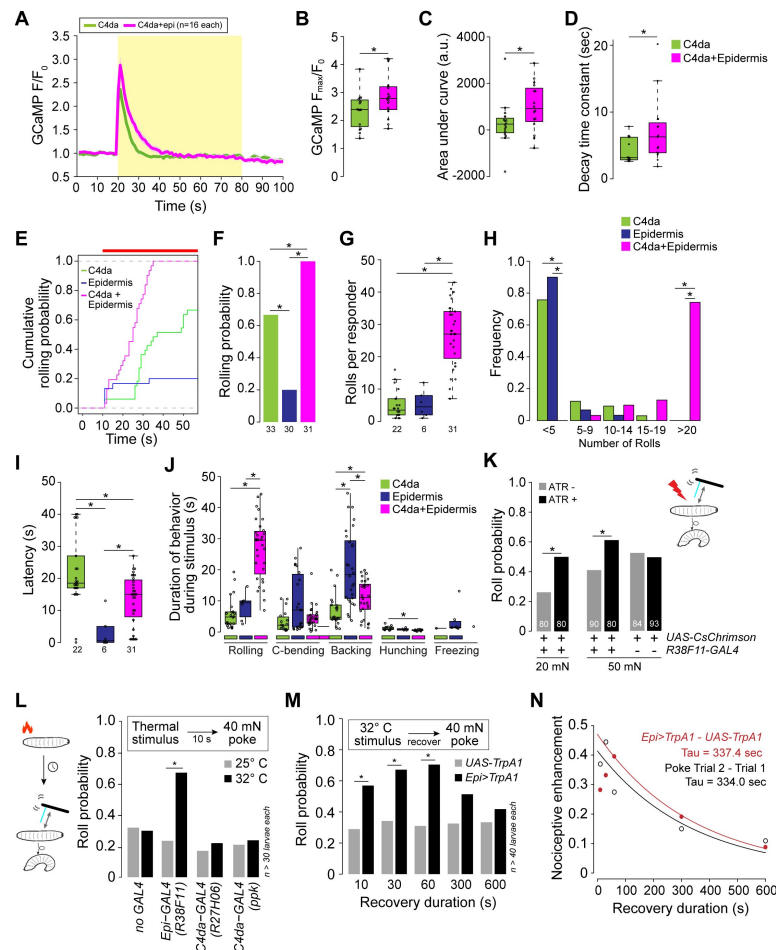


Figure 4.

Epidermal stimulation augments nociceptive responses. (A) Mean GCaMP6s responses (F/F_0) in C4da axons during optogenetic stimulation (yellow box) of C4da neurons alone (green) or of C4da neurons and epidermal cells (magenta), shading indicates SEM. (B) Simultaneous epidermal stimulation increased the peak calcium response (F_{max}/F_0), (C) total calcium influx (area under the curve), and (D) duration of C4da neuron calcium responses compared to stimulation of C4da neurons alone. Genotypes: *ppk-LexA, AOP-GCaMP6s/+; R27H06-GAL4/UAS-CsChrimson* (C4da) and *ppk-LexA, AOP-GCaMP6s/+; R27H06-GAL4/R38F11-GAL4, UAS-CsChrimson* (C4da+epi). (E-J) Characterization of the behavioral responses to low-intensity optogenetic stimulation of C4da neurons, epidermal cells, or simultaneous C4da neurons and epidermal cells. (E) Cumulative and (F) total roll probability during optogenetic stimulation (indicated by the red bar). $n = 33$ (*C4da>CsChrimson*), 30 (*Epi>CsChrimson*), and 31 (*C4da + Epi>CsChrimson*) larvae. (G, H) Number and frequency distribution of rolls, (I) latency to the first roll observed for larvae of the indicated genotypes, and (J) the duration of the indicated behaviors during light stimulus. Genotypes: *UAS-CsChrimson/+; R27H06-GAL4/+* (C4da), *UAS-CsChrimson/+; R38F11-GAL4/+* (Epidermis), *UAS-CsChrimson/+; R27H06-GAL4/R38F11-GAL4* (C4da+Epidermis). (K) Roll probability of larvae to a 20 mN or 50 mN von Frey mechanical stimulus and epidermal optogenetic activation (a light stimulus, $1.16 \mu W/mm^2$ that was insufficient on its own to induce nociceptive rolling). Larvae were reared in the presence or absence of ATR, as indicated. Genotypes: *UAS-CsChrimson/+; R38F11-GAL4/+*. (L-N) Prior epidermal but not nociceptor stimulus potentiates mechanical nociceptive responses. (L) Roll probability of control larvae (*UAS-TrpA1/+*) or larvae expressing TrpA1 in the epidermis (*Epi-GAL4: R38F11-GAL4*) or C4da neurons (*UAS-TrpA1/+; C4da-GAL4 #1: R27H06-GAL4, UAS-TrpA1/+; C4da-GAL4 #2: ppk-GAL4, UAS-TrpA1/+*), or control larvae (*no GAL4: UAS-TrpA1/+*) in response to 40 mN mechanical stimulus 10 s following 10 s of a thermal stimulus (25° or 32° C). To control for effects of genetic background, we confirmed that each of the experimental genotypes exhibited mechanically induced nociceptive sensitization (Fig. 4S1C). (M) Roll probability of control larvae (*UAS-TrpA1/+*) or larvae expressing TrpA1 in the epidermis (*Epi>TrpA1: R38F11-GAL4, UAS-TrpA1/+*) in response to a 40 mN mechanical stimulus delivered at the indicated time interval following a 32° C thermal stimulus. (N) Nociceptive enhancement (difference in the roll probability to the first and second stimulus) is plotted against the recovery duration and results were fit to an exponential curve to derive the decay time constant. The red line indicates nociceptive enhancement of a mechanical stimulus by a prior epidermal thermogenetic stimulus; the black line indicates nociceptive enhancement by a prior mechanical stimulus.

1% stretch recruited an additional 10% of stretch-responding cells (Fig. 5B–5D). Overall, 51% of epidermal cells displayed stretch sensitivity (Fig. 5C, 5D). We also found that 43% of epidermal cells responded to hypoosmotic challenge and 35% responded to laminar flow; 19% of epidermal cells responded to both hypoosmotic challenge and laminar flow (Fig. 5S1). Given that dissociated epidermal cells were intrinsically mechanosensitive, we next assessed mechanically evoked responses in a semi-intact body wall preparation (Fig. 5E). We found that 50% of epidermal cells exhibited a robust calcium transient in response to a 25 μ m membrane displacement using a glass probe (Fig. 5E–5G, 5S1G). Altogether, these results indicate that *Drosophila* larval epidermal cells are intrinsically mechanosensitive.

Mechanically evoked epidermal responses rely on store-operated calcium entry

Our studies demonstrate that, like vertebrate keratinocytes, *Drosophila* epidermal cells exhibit mechanically evoked calcium transients. What is the mechanism of mechanotransduction in these cells? RNA-seq analysis of acutely dissociated epidermal cells revealed expression of more than 20 cation channels, including the mechanosensitive ion channels Piezo, TMEM63, and TMCO (Fig. 6S1). We assessed the epidermal requirements of these channels in mechanical nociception using available RNAi transgenes (Fig. 6A). Our behavioral screen identified one channel, *Orai*, the sole *Drosophila* pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel (Feske *et al.*, 2006), that blocked mechanically-evoked nociceptive sensitization without impacting behavioral responses to the first stimulus (Fig. 6A, 6B, 6S2A) or altering nociceptor morphogenesis (Fig. 1S4). Interestingly, our screen uncovered an epidermal role for *Task6*, an orthologue of stretch-sensitive 2-pore potassium channels (Fink *et al.*, 1996), in mechanonociception, as *Task6* RNAi increased nocifensive rolling responses to the initial mechanical stimulus (Fig. 6S2B). Finally, although our RNAi studies did not reveal an epidermal requirement for other known mechanosensitive cation channels in mechanonociceptive behaviors, it is possible that multiple channels function redundantly, or that RNAi knockdown was incomplete.

To gain insight into mechanically evoked nociceptive sensitization, we focused on probing the role of *Orai* in epidermal mechanosensory responses. We first asked whether *Orai* is functional in *Drosophila* epidermal cells. *Orai* is a store-operated calcium (SOC) channel that is activated by the calcium-sensitive, endoplasmic reticulum (ER) molecule Stim, upon calcium release from ER calcium stores. Thapsigargin (TG) induces calcium release from intracellular stores and thus triggers Stim-dependent activation of *Orai* channels. Indeed, *Drosophila* epidermal cells displayed TG-induced calcium release from stores in the absence of extracellular calcium, followed by calcium influx upon re-addition of extracellular calcium (Fig. 6C). Calcium influx was significantly inhibited by the addition of low nanomolar lanthanum, consistent with the high sensitivity of *Orai* channels to lanthanides (Fig. 6S2C). This characteristic store operated calcium entry (SOCE) response was significantly reduced by epidermis-specific *Stim* or *Orai* RNAi knockdown (Fig. 6S2D–6S2F). Consistent with a key role for SOCE in mechanotransduction, we found that radial stretch in the absence of extracellular calcium induced calcium release from intracellular stores, as well as calcium influx upon re-addition of extracellular calcium. These data show that mechanically evoked responses in epidermal cells involve both ER calcium release and store-operated calcium entry. While both store release and calcium influx constitute the calcium response to stretch, in 69% of cells, calcium due to store release exceeded that of calcium re-entry (Fig. 6E). Consistent with this observation, depletion of intracellular stores and inhibition of calcium influx reduced the number of stretch sensitive cells by 61% (stretch non-responsive cells in WT = 49% vs. store depleted = 80%) and 30% (stretch non-responsive cells in WT = 49% vs. La^{3+} = 64%; Fig. 6F–G), respectively.

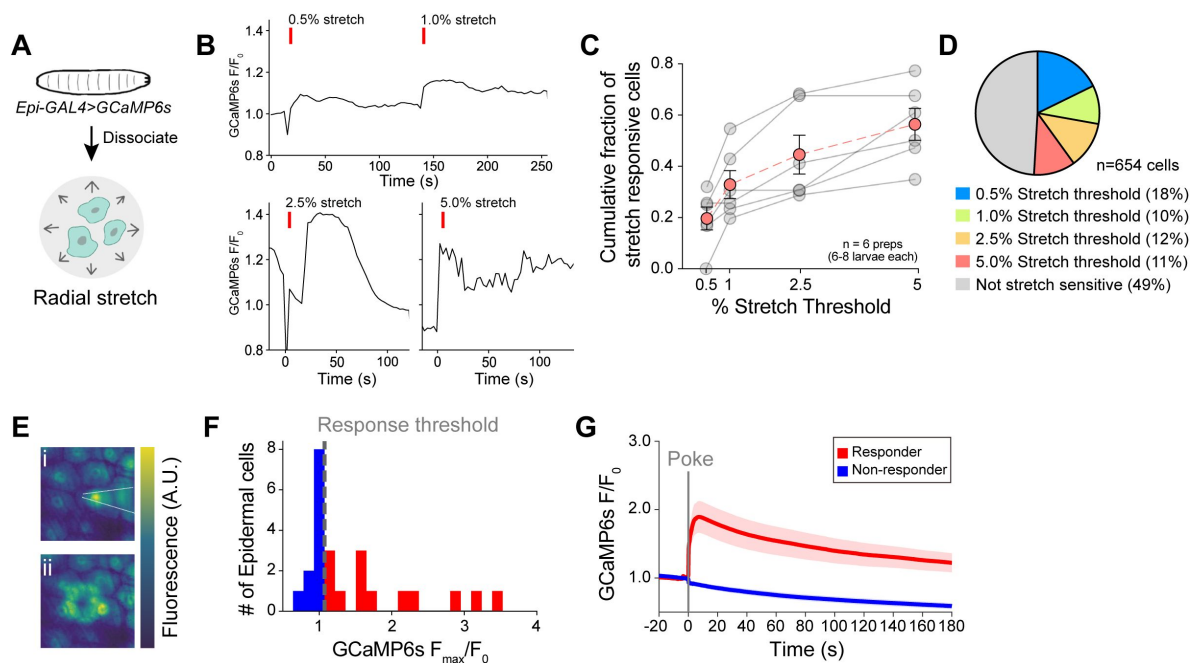


Figure 5.

Epidermal cells are intrinsically mechanosensitive. (A) Schematic of preparation to measure radial stretch evoked calcium responses of dissociated epidermal cells. (B) Representative calcium responses of a dissociated epidermal cell to 0.5% and 1% radial stretch (successive stimuli), 2.5% radial stretch, and 5% radial stretch. (C) Dose response curve displaying the fraction of epidermal cells activated by increasing magnitudes of stretch. Red trace displays the mean $\pm$ SEM across six independent dissociated cell preparations, obtained from a minimum of 6 larvae. Gray traces display fraction responding in each dissociated cell preparation replicate. (D) Subsets of epidermal cells display varying stretch thresholds, $n = 6$ dissociated cell preparations, for a total of 654 epidermal cells. (E) Representative mechanically induced epidermal calcium responses in the larval body wall. Images show GCaMP6s fluorescence intensity 100 ms prior to (i) and 20 s following (ii) a 25 μ m membrane displacement (poke). (F) Distribution of the peak calcium response (F_{max}/F_0) to a 25 μ m membrane displacement (poke) of 24 cells from 24 independent larval fillets. Cells were classified as responders (>10% increase in normalized GCaMP6s fluorescence). (G) Mean calcium responses (F/F_0) of poke responders and non-responders ($n = 12$ cells each). Solid lines depict mean normalized GCaMP6s fluorescence and shading indicates SEM. Genotype: *R38F11-GAL4, UAS-GCaMP6s*.

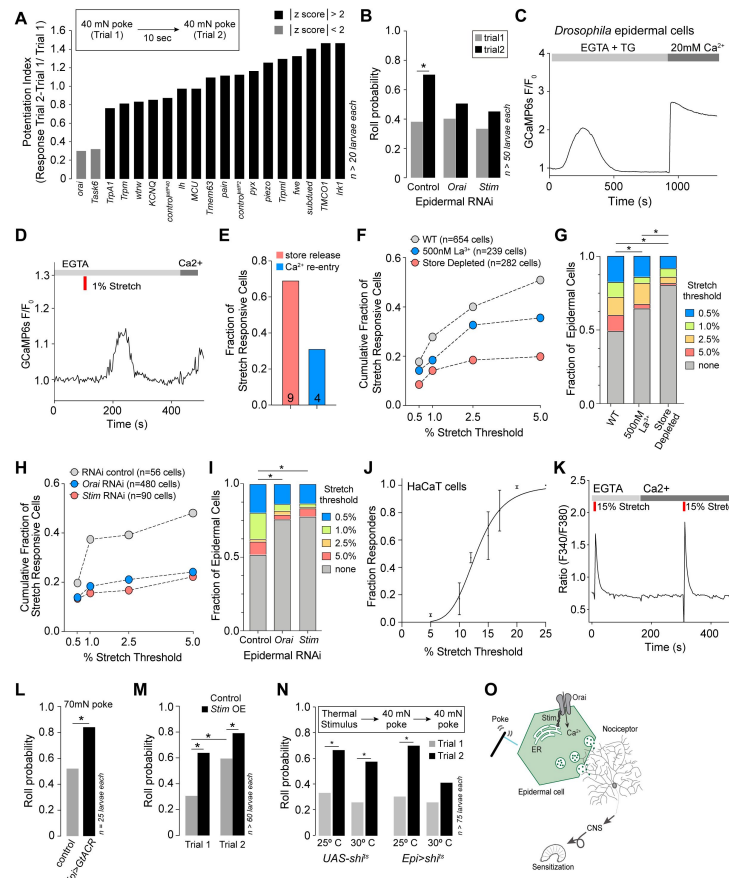


Figure 6.

CRAC channels are required for epidermal mechanosensory responses and epidermal nociceptive potentiation. (A) RNAi screen for epidermal ion channels required for mechanically induced nociceptive potentiation. Bars depict nociceptive potentiation index (difference in the larval roll probability to the first and second mechanical stimuli divided by roll probability to the first mechanical stimulus). Candidate channels were chosen for further analysis if they had a z-score greater than 2 (absolute value). (B) The CRAC channels *Orai* and *Stim* are required in epidermal cells for mechanically evoked nociceptive potentiation. Roll probability of larvae of the indicated genotypes (Control RNAi, *R38F11-GAL4, UAS-RFP-RNAi/+*; *Stim* RNAi, *R38F11-GAL4, UAS-Stim-RNAi/+*; *Orai* RNAi, *R38F11-GAL4, UAS-Orai-RNAi/+*) to a 40 mN mechanical stimulus followed by a second 40mN mechanical stimulus 10 s later. (C) *Drosophila* epidermal cells display classical store-operated calcium entry (SOCE). Treatment with the drug thapsigargin (TG) in the absence of extracellular calcium promoted depletion of intracellular calcium stores and calcium influx, following extracellular calcium re- addition. (D) Like TG, 1% stretch in the absence of extracellular calcium induced depletion of intracellular calcium stores and calcium influx, following extracellular calcium re-entry. (E) 69% of stretch responsive cells displayed greater calcium influx during intracellular calcium stores release than during the calcium re-entry phase. (F-G) The *Orai* blocker, lanthanum chloride (500 nM) or the depletion of intracellular stores by thapsigargin (1 μ M) reduces the fraction of stretch-sensitive epidermal cells. (H-I) The fraction of stretch-sensitive epidermal cells is significantly decreased in cells isolated from larvae expressing *Stim* RNAi, or *Orai* RNAi, as compared to control RNAi. (J) Stretch stimuli evoke dose-dependent calcium signals in the human keratinocyte HaCaT cell line. (K) Representative stretch evoked SOCE calcium response in HaCaT cells. Stretch induces calcium release from stores in the absence of extracellular calcium and a greater calcium influx in the presence of extracellular calcium. (L) Epidermal hyperpolarization enhances mechanical nocifensive responses. Roll probability of larvae expressing GtACR in epidermal cells (*R38F11-GAL4, UAS-GtACR/+*) or control larvae (*R38F11-GAL4/+*) to a single 70 mN mechanical stimulus. (M) Epidermal *Stim* overexpression enhances mechanical nocifensive responses. Roll probability of *Stim*- overexpressing larvae (*R38F11-GAL4, UAS-Stim/+*) and control larvae (*R38F11-GAL4/+*) to two successive 40 mN mechanical stimuli delivered 10 s apart. (N) Epidermal potentiation of mechanical nociceptive responses requires exocytosis. Roll probability of control larvae (*UAS-shi^{TS}/+*) or larvae expressing temperature-sensitive dominant-negative *shi* in epidermal cells (*R38F11-GAL4, UAS-shi^{TS}/+*) in response to two successive mechanical stimuli that followed 10 min of conditioning at the permissive (25° C) or non-permissive (30° C) temperature. (O) Model of epidermal-neuronal signaling. Mechanically evoked *Stim/Orai* calcium signaling in epidermal cells drives calcium influx and vesicle release that drives nociceptor activation and mechanical sensitization via activation of C4da nociceptors.

Given that Stim and Orai mediate SOCE, we investigated requirements for epidermal Stim and Orai in mechanically evoked calcium responses. RNAi knockdown of either *Stim* or *Orai* significantly reduced the fraction of stretch-responsive epidermal cells (RNAi control = 48%, *Stim* RNAi = 22%, *Orai* RNAi = 24%; **Fig. 6H-I**), with *Stim* or *Orai* RNAi preferentially attenuating stretch evoked responses to larger magnitude stretch stimuli. We also found that human keratinocytes display dose-dependent stretch evoked calcium responses, though they respond to higher magnitudes of stretch than *Drosophila* epidermal cells (**Fig. 6J**). Like *Drosophila* epidermal cells, both ER calcium release and store-operated calcium entry constitute the mechanically-evoked calcium responses in human keratinocytes (**Fig. 6K**).

Two hallmarks of Orai channels are steep inward rectification, with larger currents at hyperpolarizing potentials, and highly cooperative Orai activation by Stim (Hoover and Lewis, 2011). Since Stim and Orai mediate mechanical responses of epidermal cells *in vitro*, we predicted that increasing the calcium driving force through Orai activity by either hyperpolarizing *Drosophila* epidermal cells or by activating additional Orai channels via *Stim* overexpression would enhance behavioral responses to mechanical stimuli. Indeed, we found that hyperpolarizing epidermal cells with the light-activated anion channelrhodopsin GtACR1 (Mohammad *et al.*, 2017) increased behavioral responses to mechanical stimuli (**Fig. 6L**). In addition, overexpressing *Stim* in epidermal cells significantly enhanced nocifensive behavioral responses to mechanical stimuli (**Fig. 6M**). Altogether, these results demonstrate that mechanically evoked responses of epidermal cells and the resulting nocifensive behavior outputs require store-operated calcium entry.

How might mechanically evoked calcium entry in epidermal cells drive nociceptor activation and behavior? Stim/Orai-mediated calcium entry contributes to exocytosis in a variety of cell types, including neurons and immune cells (Pores-Fernando and Zweifach, 2009; Ashmole *et al.*, 2012; Maneshi *et al.*, 2020; Chanaday *et al.*, 2021; Ramesh *et al.*, 2021). Therefore, we investigated the contribution of epidermal exocytosis in nociceptive sensitization with the temperature-sensitive dynamin mutant *shibire^{ts}* (*shi^{ts}*) to inducibly block vesicle recycling, as this treatment rapidly and potently blocks neurotransmitter release (Koenig *et al.*, 1983) and we found that acute epidermal dynamin inactivation using *UAS-shi^{ts}* had no discernable effect on nociceptor morphogenesis (Fig. 1S4). In this paradigm, larvae expressing *shi^{ts}* in epidermal cells, but not control larvae, exhibited significant attenuation of mechanically evoked nociceptive sensitization following pre-incubation at the non-permissive temperature (**Fig. 6N**). In contrast, both genotypes exhibited comparable responses to a mechanical stimulus at the permissive temperature (25° C) and to the first mechanical stimulus following pre-incubation at the non-permissive temperature (30° C). Taken together, these results are consistent with a model in which mechanical stimuli induce calcium influx and vesicular release from epidermal cells, which in turn activates nociceptors to induce acute nocifensive behaviors and prolonged sensitization (**Fig. 6O**). Although our RNA-seq analysis of epidermal cells did not reveal expression of neurotransmitter biosynthesis genes, epidermal cells express a large repertoire of genes involved in vesicular release as well as several neuropeptide genes, providing an entry point to defining the molecules involved in epidermis-SSN communication (Fig. 6S3).

Discussion

In this study, we have shown an essential role for *Drosophila* epidermal cells in escape responses to noxious mechanical stimuli. Activation of epidermal cells acutely activates SSNs to induce an array of behavioral outputs and mechanical sensitization. This epidermal potentiation persists for minutes to promote a prolonged, but reversible, mechanical hypersensitivity that may protect from further insult. This is distinct from previously described forms of neuropathic thermal and mechanical hypersensitivity in *Drosophila* which are induced by tissue damage and chemotherapeutic agents, respectively, emerge on a timescale of hours, and are long-lasting

(Babcock *et al.*, 2009 [DOI](#); Boiko *et al.*, 2017 [DOI](#); Khuong *et al.*, 2019 [DOI](#)). In the mammalian somatosensory system, a variety of inflammatory mediators have been shown to activate TRPA1 in neurons to promote mechanical hypersensitivity (Bautista *et al.*, 2006 [DOI](#)); however, the molecular force transducers that mediate mechanical pain are unknown. In contrast, in the *Drosophila* somatosensory system, *Ppk1/Ppk26*, *Piezo*, and *Trpa1* are key transducers of mechanonociception (Zhong *et al.*, 2010 [DOI](#); Kim *et al.*, 2012b [DOI](#); Gorczyca *et al.*, 2014 [DOI](#); Guo *et al.*, 2014 [DOI](#); Mauthner *et al.*, 2014 [DOI](#)). Prolonged sensitization to noxious mechanical stimuli plays an important protective role in an organism's survival; yet the mechanisms of mechanical sensitization of *Drosophila* nociceptors were unknown.

We demonstrate a new role for SOC signaling in both *Drosophila* and human epidermal cell mechanotransduction. While short-term sensitization is beneficial to survival, a key hallmark of pathological pain is prolonged and persistent mechanical hypersensitivity; whether deregulation of this mechanism of epidermis-evoked short-term sensitization contributes to pathological pain remains to be determined. Overall, we identified a mechanism that does not impact acute nociception but selectively regulates mechanical sensitization. These findings highlight Stim/Orai signaling as a new avenue for understanding mechanical pain.

This work has opened several new directions for future studies. First, how does radial and osmotic stretch lead to the activation of store-operated calcium signaling? Although Orai has not previously been shown to be mechanosensitive, our studies revealed a requirement for Orai and its activator Stim in mechanically-evoked calcium flux in *Drosophila* epidermal cells. We also showed that radial stretch of human keratinocytes triggered both calcium release from stores and SOCE; our previous studies showed that Stim and Orai are required for SOCE in human keratinocytes (Wilson *et al.*, 2013 [DOI](#)). These data, in combination with other studies showing that mechanical stimulation of human mesenchymal stem cells and mouse enteroendocrine cells (Knutson *et al.*, 2023; Kim *et al.*, 2015 [DOI](#); Knutson *et al.*, 2023 [DOI](#)) also triggers SOCE suggests that Stim/Orai signaling may represent a conserved pathway for mechanotransduction in non-neuronal cells.

Second, how is Stim/Orai function linked to mechanotransduction? Stim/Orai signaling is activated downstream of G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) through phospholipase C. Studies have shown that a number GPCRs are mechanosensitive (Chachisvilis *et al.*, 2006 [DOI](#); Grosmaître *et al.*, 2007 [DOI](#); Mederos y Schnitzler *et al.*, 2008 [DOI](#); Connelly *et al.*, 2015 [DOI](#); Xu *et al.*, 2018 [DOI](#)). Indeed, this mechanism has been proposed for mechanically evoked enteroendocrine activation in the gut epithelium (Knutson *et al.*, 2023 [DOI](#)), though this has not been studied in epidermal cells. Alternatively, plasma membrane deformation has been shown to induce formation of ER-plasma membrane junctions (Venturini *et al.*, 2020 [DOI](#); Aoki *et al.*, 2021 [DOI](#)), where Stim and Orai clusters accumulate and interact to drive calcium influx (Luik *et al.*, 2008 [DOI](#)).

Finally, a recent paper demonstrated that mechanical stimulation of the ER membrane itself promotes calcium release from ER stores via the opening of calcium-permeable ion channels in the ER membrane (Song *et al.*, 2024 [DOI](#)).

Third, how does mechanically induced signaling in epidermal cells lead to modulation of SSNs? Our data support a model whereby epidermal cells and multiple classes of SSNs are functionally coupled. Epidermal stimulation modulates activity of nociceptive C4da neurons, mechanosensory C3da and Cho neurons, and proprioceptive C1da neurons, and the output of neuronal activity is required for epidermis-evoked behaviors. We demonstrated a requirement for dynamin-dependent vesicle release from epidermal cells in mechanical sensitization, providing a potential link between Stim/Orai signaling in epidermal cells and downstream neuronal activity. However, the mediators that are released by epidermal cells and the signaling molecules in the nociceptors remain unknown. Furthermore, whether different types of SSNs are coupled to epidermal cells by distinct mechanisms remains to be determined. At least in the case of Cho neurons which are

wrapped by ensheathing glial cells and scolopale cells, signaling from epidermal cells likely involves at least one additional cell type. Finally, we find that epidermal cells exhibit a dose-dependent response to radial stretch; we therefore anticipate that the output of epidermal cells is likewise dependent on the stimulus intensity. Hence, rather than a fixed threshold beyond which epidermal cells are selectively activated, we hypothesize that increasing stimulus intensities drive increasing signal outputs to neurons.

Epidermal cells ensheath peripheral arbors of some SSNs, including *Drosophila* nociceptive C4da neurons and, to a lesser extent, mechanosensory C3da neurons (Jiang *et al.*, 2019). Hence, epidermal sheaths could facilitate transduction of epidermal signals that modulate nociceptor function. Consistent with this possibility, blocking ensheathment attenuates *Drosophila* larval responses to noxious mechanical stimuli (Jiang *et al.*, 2019) and likewise impairs function of some *C. elegans* mechanosensory neurons (Chen and Chalfie, 2014). However, our finding that epidermal stimulation evokes calcium responses from SSNs that are not ensheathed by epidermal cells (C1da, Cho neurons) argues that epidermal sheaths are unlikely to play an essential function in epidermis-SSN functional coupling. Instead, ensheathment may facilitate nociceptor activation by increasing the efficiency of vesicular exchange or, alternatively, may modulate nociceptor activity through enhanced ionic coupling to epidermal cells.

Which epidermal-derived molecules might modulate neuronal activity? There are several mechanisms by which mammalian epidermal cells activate SSNs. Vesicular release of norepinephrine from mouse epidermal Merkel cells is required for sustained touch-evoked firing of mechanosensory neurons (Hoffman *et al.*, 2018). Additionally, mechanical stimuli trigger ATP release from mouse keratinocytes that activates nociceptors via purinergic (P2X4) receptors (Koizumi *et al.*, 2004; Tsutsumi *et al.*, 2009; Moehring *et al.*, 2018). Finally, Stim/Orai-dependent SOCE mediates the release of the cytokine thymic stromal lymphopoietin (TSLP) from epidermal keratinocytes that directly activates a subset of TRPA1-expressing SSNs to induce itch (Wilson *et al.*, 2013).

Similar to these mammalian models, UV-damage has been shown to induce the release of the cytokine Eiger to promote *Drosophila* nociceptor sensitization (Babcock *et al.*, 2009); though this occurred on a slower timescale than the epidermal-evoked mechanical sensitization we describe here (8 h vs. ~ 10 sec, Fig. 4L). Likewise, epidermal platelet-derived growth factor (PDGF) ligands regulate mechanonociceptive responses in *Drosophila* (Lopez-Bellido *et al.*, 2019) and intrathecal delivery of PDGF or the closely related growth factor EGFR yields mechanical hypersensitivity in rats (Masuda *et al.*, 2009; Puig *et al.*, 2020), but it remains to be determined whether growth factor signaling can yield rapid sensitization. Hence, future studies will address which neurotransmitters, neuropeptides, or inflammatory mediators underlie epidermal cell-mediated mechanical sensitization.

Our data support a model whereby epidermal cells and multiple classes of SSNs are functionally coupled. Future studies will address which neurotransmitters, neuropeptides, or inflammatory mediators underlie epidermal cell-mediated mechanical sensitization. An additional key next step is understanding whether the neuronal plasticity underlying mechanical sensitization results from the direct modulation of mechanosensitive channels or rapid insertion of new mechanosensitive channels into the plasma membrane, or from changes in the signaling pathways or channels that regulate neuronal excitability. Overall, we performed an unbiased genetic screen that for the first time establishes a key role for mechanically evoked Stim/Orai calcium signaling in epidermal cells that drive nociceptor modulation and mechanical hypersensitivity.

Material and methods

Materials availability and community standards

Raw sequencing reads and gene expression estimates are available in the NCBI Sequence Read Archive (SRA) and in the Gene Expression Omnibus (GEO) under accession number GSE284380. Raw data used for analyses in this study is presented in the supplementary materials as Source Data and details of statistical analyses are presented in Supplementary File 1. ICMJE guidelines were used to define authorship roles and the ARRIVE essential 10 guidelines were used for the reporting of our in vivo studies.

Drosophila strains

Flies were maintained on standard cornmeal-molasses-agar media and reared at 25° C under 12 h alternating light-dark cycles. For all experiments involving optogenetic manipulations, larvae were raised in the constant dark at 25 °C on Nutri-Fly Instant Food (Genesee Scientific #66-117), supplemented with 1 mM all-trans retinal (ATR; Sigma #R2500). A complete list of alleles used in this study is provided in the Key Resources Table. Experimental genotypes are listed in figure legends.

Cell lines

A human keratinocyte cell line (HaCaT) was used in this study. HaCaT cells were obtained from Cyton (Sioux Falls, SD), who performed STR authentication and mycoplasma-free certification.

Behavior analysis

Optogenetic behavior screen

Individual larvae were rinsed in ddH₂O, transferred to an agarose substrate (1% agarose, 100 mm dish) in a darkened arena, and habituated for 30 s. Larvae were stimulated with a top-mounted 488 nm LED illuminator (PE-300, CoolLED) and images were captured with a sCMOS camera (Orca Flash 3.0, Hamamatsu) at frame acquisition rate of 20 fps and behaviors were scored before, during and after optogenetic stimulation.

High resolution video tracking of optogenetic-gated larval behavior

Following 5 min of light deprivation including 15 s of habituation in the behavioral arena, larvae were tracked before, during and after optical stimulus (10 s each, 30 s total) (**Fig. 2A**). For these studies we modified our stimulation paradigm in two key ways: to avoid potential contributions of nociceptor light evoked responses (Xiang *et al.*, 2010), we stimulated larvae using yellow-shifted light; and to facilitate kinetic analysis of behavior outputs, we used an automated shutter. Larvae were stimulated with a top-mounted 585 nm LED illuminator (SPECTRA X, Lumencor) equipped with a filter (FF01 585/40-25, Semrock), and images were captured with a sCMOS camera (Zyla4.2, Andor) at a frame rate of 20Hz. Larvae were constantly illuminated with an infrared (940 nm) light source (LDR2-132IR2-940-LA, CSS) for visualization. Larvae were fed (ATR+) or vehicle alone (ATR-) as indicated. Illumination intensities for optogenetic behavior studies were: 300 $\mu\text{W}/\text{mm}^2$ for Fig. 1S6, **Fig. 2B–2E**, Fig. 2S1, **Fig. 3E–3F**, Fig. 3S2A–3S2C; 25 $\mu\text{W}/\text{mm}^2$ for **Fig. 4E–4J**; 1.16 $\mu\text{W}/\text{mm}^2$ for **Fig. 4K**. Annotated videos showing responses of representative larvae to optogenetic epidermal and nociceptor stimulation are provided in **Figure 2** – movie 1 and 2.

Thermogenetic behavior assays

Larvae for thermogenetic assays were reared at room temperature (20° C) to limit TRPA1 activation during development. Third instar larvae were isolated from their food, washed in distilled water, and recovered to damp agar plates for several min, and transferred individually to a Peltier plate held at 25° C or 35° C. Behavior responses were recorded under infrared light with a computer-controlled GigE camera (FLIR) at an acquisition rate of 20 fps for 20 s. Responses were analyzed post-hoc blind to genotype and were plotted as the proportion of larvae that exhibited at least one complete nocifensive roll during stimulus application.

Mechanonociception assays

Third instar larvae were isolated from their food, washed in distilled water, and placed on a scored 35 mm petri dish with a thin film of water such that larvae stayed moist but did not float. Larvae were stimulated dorsally between segments A4 and A7 with calibrated Von Frey filaments that delivered the indicated force upon buckling, and nocifensive rolling responses were scored during the 10 s following stimulus removal. For assays involving multiple stimuli, larvae were stimulated individually, allowed to freely locomote in the arena for up to 1 min (for longer recoveries larvae were recovered onto 2% agar to prevent desiccation), and subsequently presented with the second stimulus. For assays involving thermal and mechanical stimuli, larvae were individually transferred to a pre-warmed Peltier plate containing a thin layer of water, incubated for the indicated time, and transferred to the behavior arena (or a 2% agar plate for recoveries > 1 min) with a paint brush for subsequent mechanical stimulation. For assays involving optical and mechanical stimuli, larvae were raised in constant dark at 25 °C on food supplemented with 1 mM all-trans retinal (detailed above), transferred to the behavior arena with 25 $\mu\text{W}/\text{mm}^2$ broad spectrum illumination, and assayed for responses to mechanical stimuli. All assays were conducted in ambient light except for experiments with GtACR (Fig. S72), which were conducted under 500-700 nm LED illumination (CoolLED PE-300, green). Our illumination setup for these experiments provided limited working distance, therefore larvae were restrained with forceps and given only a single stimulus.

Video annotations

Videos of individual larvae responding to light stimuli were scored on a frame-by-frame basis using the annotation software BORIS (Friard and Gamba, 2016 [DOI](#)). Behaviors scored, along with descriptions of the criteria for each behavior, are detailed in Table S1. Video analysts were blind to the genotype and treatment during scoring. Scoring on a training set was compared across all analysts to calibrate, and any behaviors for which the primary analyst was uncertain were reviewed by an additional analyst.

Additionally, 10% of videos were scored independently by two analysts and there was at least 80% concordance in behaviors annotated in these comparisons.

Microscopy

Calcium imaging: ventral nerve cords

Third-instar larvae were dissected along the dorsal midline and pinned on a sylgard- coated dish (Sylgard 184, Dow Corning). The internal organs except for neural tissues were removed. Larvae were bathed in HL3.1 (Feng et al, 2004 [DOI](#)) modified to remove calcium (Table S2) to minimize larval movement. The ventral nerve cord was imaged using an Olympus BX51WI microscope, equipped with a spinning-disk confocal unit Yokogawa CSU10 (Yokogawa) and an EM-CCD digital camera (Evolve, Photometrics). For activation of epidermal cells with the light gated CsChrimson, red light was delivered by a pE-300 (CoolLED) equipped with a filter (ET645/30x, Chroma) at a light

intensity of 30 $\mu\text{W}/\text{mm}^2$. Obtained images were analyzed using Metamorph (<https://www.moleculardevices.com/systems/metamorph-research-imaging>) and ImageJ (Schneider *et al.*, 2012). Baseline fluorescence was calculated as the mean fluorescence intensity of an ROI over the ten frames prior to light stimulus delivery. The trapezoidal method was used to calculate area under the curve, utilizing the trapz function of MATLAB. Data points from the onset of stimulation to the end of stimulation were used for the calculation.

Calcium imaging: fillet preparations

Third-instar larvae were dissected along the ventral midline and pinned on sylgard (Dow Corning) dishes with the internal surface facing towards the microscope. All internal organs, including the central nervous system, were removed. Larvae were bathed in calcium-containing HL3.1 (Feng *et al.*, 2004) (Table S2) except where indicated and images of the dorsal midline between abdominal segments A2 and A4 were captured with a Zeiss Axio Zoom V16 microscope. Captured images were analyzed using γ (Schneider *et al.*, 2012). Mechanical stimulus: fillets were poked with a tapered borosilicate capillary with a rounded tip, using a micromanipulator to induce a deflection of 25 μm . The decay time constant was calculated by fitting the data points from the peak response to the end of the experiment into an exponential curve $f(x) = a \cdot \exp(b \cdot x)$ using MATLAB with $R^2 > 0.9$ used as a threshold for reliable fitting.

Calcium imaging: dissociated epidermal cells

Six to eight larval fillets were dissociated in 400 μL of 50% Saline (modified Ringer's recipe) / 50% Schneider's media with 200 U/mL collagenase type I (Fisher 17-100-017), with mixing at 1000 RPM at 33°C for 16 min, with trituration every 8 min. Undigested fillets were removed and the remaining suspension was spun at 500 g for 3 min, followed by aspiration of the supernatant down to a 10 μL cell suspension. Cells were resuspended in 30 μL fresh PBS / Schneider's solution and plated onto poly-D-lysine (1 mg/ml, Sigma P7886) coated No. 1 coverslips, with 10 μL cell solution per coverslip.

Cells were cultured at least 30 min and up to 2 h at 25° C prior to imaging. Cells were imaged using a 10x objective at a frame rate of 0.33 Hz. Solutions are indicated in figure legends (see Table S2 for recipes). Obtained images were analyzed using MetaFluor and Python and baseline fluorescence was calculated as the mean fluorescence intensity of an ROI over 5 frames prior to stimulus delivery. For stretch stimulation, circular membranes were cut with an arch punch from sheets of glossy silicone of 0.01–0.02 inch thickness (Specialty Manufacturing, Inc.) and coated with 1 mg/ml poly-D-lysine for 1 h before plating cells. Membranes were mounted onto the StageFlexer system and vacuum pressure was applied through the FX-3000 system (Flexcell). Calibrations were performed using fluorescent beads attached to the membranes, and images were taken before and during a static stretch. To stimulate cells, a 2 s square wave of vacuum pressure was applied. Cells were imaged with an Olympus BX61WI upright microscope. For store-operated calcium entry measurements and osmotic stimulation, cells were imaged using a Zeiss Observer inverted microscope and solutions were perfused using the Automate Scientific ValveLink 8.2 perfusion system. At the end of each imaging session, 1 μM ionomycin was perfused and only cells that showed a calcium response, as defined by a 10% increase from baseline fluorescence, were used in analysis. Flow, osmotic and radial stretch responders were defined by a 5% increase from baseline fluorescence.

Calcium imaging: human keratinocytes

Immortalized human keratinocytes (HaCaT) cells (Cyton) were plated on silicone membranes one day prior to stretch experiments. Prior to the radial stretch experiments, cells were loaded with 1 μM Fura-2AM supplemented with 0.01% Pluronic F-127 (w/v, Life Technologies) in a physiological Ringer's solution containing the following (in mM): 140 NaCl, 5 KCl, 10 HEPES, 2 CaCl₂, 2 MgCl₂,

and 10 d-(+)-glucose, pH 7.4. Acquired images were displayed as the ratio of 340 nm/380 nm. Cells that had a response 10 standard deviations above baseline to ionomycin were included in the analysis and stretch responses were defined by a 15% increase in Fura-2 340/380 ratio.

Confocal Microscopy

For peripheral imaging of cellular morphology, live single larvae were mounted in 90% glycerol under a coverslip and imaged on a Leica SP5 confocal microscope using a 40x1.25 NA lens. To image the larval CNS, larvae were dissected on sylgard plates, briefly fixed in 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, washed 3 x 5 min in PBS, and mounted for imaging.

RNA-Seq analysis of epidermal cells

RNA isolation for RNA-Seq

Larvae with cytoplasmic GFP expressed in different epidermal subsets were microdissected and dissociated in collagenase type I (Fisher 17-100-017) into single cell suspensions, largely as previously described (Williams *et al.*, 2016 [DOI](#)), with the addition of 1% BSA to the dissociation mix. After dissociation, cells were transferred to a new 35 mm petri dish with 1 mL 50% Schneider's media, 50% PBS supplemented with 1% BSA. Under a fluorescent stereoscope, individual fluorescent cells were manually aspirated with a glass pipette into PBS with 0.5% BSA, and then serially transferred until isolated without any additional cellular debris present. Ten cells per sample were aspirated together, transferred to a mini-well containing 3ul lysis solution (0.2 % Triton X-100 in water with 2 U / μ L RNase Inhibitor), lysed by pipetting up and down several times, transferred to a microtube, and stored at -80° C. For the picked cells, 2.3 μ L of lysis solution was used as input for library preparation.

RNA-Seq library preparation

RNA-Seq libraries were prepared from the picked cells following the Smart-Seq2 protocol for full length transcriptomes (Picelli *et al.*, 2014 [DOI](#)). To minimize batch effects, primers, enzymes, and buffers were all used from the same lots for all libraries.

Libraries were multiplexed, pooled, and purified using AMPure XP beads, quality was checked on an Agilent TapeStation, and libraries were sequenced as 51-bp single end reads on a HiSeq4000 at the UCSF Center for Advanced Technology.

RNA-Seq data analysis

Reads were demultiplexed with CASAVA (Illumina) and read quality was assessed using FastQC (<https://www.bioinformatics.babraham.ac.uk/> [DOI](#)) and MultiQC (Ewels *et al.*, 2016 [DOI](#)). Reads containing adapters were removed using Cutadapt version 2.4 (Martin, 2011) and reads were mapped to the *D. melanogaster* transcriptome, FlyBase genome release 6.29, using Kallisto version 0.46.0 (Bray *et al.*, 2016 [DOI](#)) with default parameters. AA samples were removed from further analysis for poor quality, including low read depth (< 500,000 reads), and low mapping rates (< 80%). Raw sequencing reads and gene expression estimates are available in the NCBI Sequence Read Archive (SRA) and in the Gene Expression Omnibus (GEO) under accession number GSE284380.

Statistical analysis

For each experimental assay control populations were sampled to estimate appropriate sample numbers to allow detection of ~33% differences in means with 80% power over a 95% confidence interval. Details of statistical tests including treatment groups, sample numbers (which correspond

to independent biological replicates), statistical tests, p-values and q-values are provided in Supplementary File 1.

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Additional information

Author Contributions

Conception and design: Optogenetic behavior analysis, K.E., J.Z.P, C.R.W, J.Y.; larval behavior assays, J.Z.P, C.R.W, and J.Y.; in vivo calcium imaging, K.E., J.Z.P, C.R.W, J.Y.; ex vivo calcium imaging, D.M.B., S.S.M., and J.Z.P

Acquisition of data: Optogenetic behavior screen, C.J.A, C.E.E., S.M., and J.Y.; Optogenetic behavior assays, J.Y., C.H., M.M., K.I., and C.R.W.; mechanonociception assays, C.R.W, and J.Z.P; thermogenetic behavior assays, J.Z.P.; RNA-sequencing, J.Z.P. and C.R.W.

Analysis and Interpretation of data: larval behavior assays, J.Z.P, C.R.W, J.Y.; in vivo calcium imaging, K.E., J.Z.P, C.R.W, J.Y.; ex vivo calcium imaging, D.M.B. and S.S.M.; transcriptomic data, C.H., C.R.W., and J.Z.P.

Drafting the article: D.M.B., J.Z.P, C.R.W, S.S.M., and J.Y.

Additional files

14 supplemental figures, 2 supplemental tables, details of statistical analysis, and key resources table [🔗](#)

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Reviewer #1 (Public review):

Summary:

In this meticulously conducted study, the authors show that *Drosophila* epidermal cells can modulate escape responses to noxious mechanical stimuli. First, they show that activation of epidermal cells evokes many types of behaviors including escape responses. Subsequently, they demonstrate that most somatosensory neurons are activated by activation of epidermal cells, and that this activation has a prolonged effect on escape behavior. *In vivo* analyses indicate that epidermal cells are mechanosensitive and require stored-operated calcium channel Orai. Altogether, the authors conclude that epidermal cells are essential for nociceptive sensitivity and sensitization, serving as primary sensory noxious stimuli.

Strengths:

The manuscript is clearly written. The experiments are logical and complementary. They support the authors' main claim that epidermal cells are mechanosensitive and that epidermal mechanically evoked calcium responses require the stored-operated calcium channel Orai. Epidermal cells activate nociceptive sensory neurons as well as other somatosensory neurons in *Drosophila* larvae, and thereby prolong escape rolling evoked by mechanical noxious stimulation.

Weaknesses:

In several places the text is unclear. For example, core details are missing in the protocols, including the level of LED intensity used, which are necessary for other researchers to reproduce the experiments. Secondly, the rationales are missing for some experiments (for experiments X, Y, and Z). It would be helpful to clarify for your readers why the experiments (for example Figure 3S2) were performed. Finally, for most experiments, the epidermal cells are activated for 60 s, which is long when considering that nocifensive rolling occurs on a timescale of milliseconds. It would be informative to know the shortest duration of epidermal cell activation that is sufficient for observing the behavioral phenotype (prolongation of escape behavior) and activation of sensory neurons.

<https://doi.org/10.7554/eLife.95379.2.sa2>

Reviewer #2 (Public review):

Summary:

The authors provide compelling evidence that stimulation of epidermal cells in *Drosophila* larvae results in the stimulation of sensory neurons that evoke a variety of behavioral

responses. Further, the authors demonstrate that epidermal cells are inherently mechanoresponsive and implicate a role for store-operated calcium entry (mediated by Stim and Orai) in the communication to sensory neurons.

Strengths:

The study represents a significant advance in our understanding of mechanosensation. Multiple strengths are noted. First, the genetic analyses presented in the paper are thorough with appropriate consideration to potential confounds. Second, behavioral studies are complemented by sophisticated optogenetics and imaging studies. Third, identification of roles for store-operated calcium entry is intriguing. Lastly, conservation of these pathways in vertebrates raise the possibility that the described axis is also functional in vertebrates.

Weaknesses:

The study has a few conceptual weaknesses that are arguably minor. The involvement of store-operated calcium entry implicates ER calcium store release. Whether mechanical stimulation evokes ER calcium release in epidermal cells and how this might come about (e.g., which ER calcium channels, roles for calcium-induced calcium release etc.) remains unaddressed. On a related note, the kinetics of store-operated calcium entry is very distinct from that required for SV release. The link between SOC and epidermal cells-neuron transmission is not reconciled. Finally, it is not clear how optogenetic stimulation of epidermal cells results in the activation of SOC.

Revised manuscript:

The authors have adequately addressed my original concerns.

<https://doi.org/10.7554/eLife.95379.2.sa1>

Author response:

The following is the authors' response to the original reviews

Reviewer #1 (Public Review):

Summary.

In this meticulously conducted study, the authors show that Drosophila epidermal cells can modulate escape responses to noxious mechanical stimuli. First, they show that activation of epidermal cells evokes many types of behaviors including escape responses. Subsequently, they demonstrate that most somatosensory neurons are activated by activation of epidermal cells, and that this activation has a prolonged effect on escape behavior. In vivo analyses indicate that epidermal cells are mechanosensitive and require stored-operated calcium channel Orai. Altogether, the authors conclude that epidermal cells are essential for nociceptive sensitivity and sensitization, serving as primary sensory noxious stimuli.

Strengths.

The manuscript is clearly written. The experiments are logical and complementary. They support the authors' main claim that epidermal cells are mechanosensitive and that epidermal mechanically evoked calcium responses require the stored-operated calcium channel Orai. Epidermal cells activate nociceptive sensory neurons as well as other somatosensory neurons in Drosophila larvae, and thereby prolong escape rolling evoked by mechanical noxious stimulation.

Weaknesses.

Core details are missing in the protocols, including the level of LED intensity used, which are necessary for other researchers to reproduce the experiments. For most experiments, the epidermal cells are activated for 60 s, which is long when considering that nocifensive rolling occurs on a timescale of milliseconds. It would be informative to know the shortest duration of epidermal cell activation that is sufficient for observing the behavioral phenotype (prolongation of escape behavior) and activation of sensory neurons.

(1) We agree with the reviewer that the LED intensity is an important detail of the experimental paradigm. We updated the methods to include intensity measurements for the stimuli used throughout the manuscript.

(2) The Reviewer asks about the shortest duration of epidermal cell activation sufficient for observing the behavior phenotype. We note in the manuscript that behavioral responses to optogenetic epidermal stimulation are apparent within 2 seconds of stimulus (see Figure 2F); this is consistent with our calcium imaging data in which C4da response reaches its maximum within 2-3 sec of stimulation.

Reviewer #1 (Recommendations):

(1) The epidermal cells in this study are activated for 60 s. In the real world, the nociceptive stimulation (a poke, such as penetration by the ovipositor of a parasitic wasp) that evokes escape rolling is short. Does optogenetic activation of 1 s or less still evoke rolling? For example, it is unclear in Figure 4K how long the epidermal cells need to be activated before the poke stimulus prolongs rolling. Is it possible to test behavior and GCaMP activity in sensory neurons when epidermal cells are briefly (1 second) activated?

As described above, behavioral responses to optogenetic epidermal stimulation are apparent within 2 seconds of stimulus (see Figure 2F); this is consistent with our calcium imaging data in which C4da response reaches its maximum within 2-3 sec of stimulation. The kinetics are consistent with a role for epidermal cells in modulating neuronal responses to nocifensive stimuli, and similar to the response kinetics observed in mammalian epidermal cells that modulate neuronal touch and pain responses (Maksimovic et al., 2014; Woo et al., 2014; Mikesell et al., 2022).

(2) The protocol for optogenetic screening states that the authors used a 488-nm LED. Why was a 488-nm LED used instead of the 610-nm LED for Chrimson activation? No information (except figure 4K) about the light intensity is provided in the figure legend or the protocol section. Please state the LED intensity used for all optogenetic experiments (GCaMP imaging, behavioral experiments, etc.).

We used 488 nm light for the initial screen for technical reasons. The screen was conducted by students at the MBL Neurobiology course (hence the affiliation; student authors are included in the manuscript), and the only LED available to us at that time delivered insufficient illumination at longer wavelengths to be useful. We chose to include the student's data because (1) we found that the 488 nm light alone did not induce rolling in our setup, (2) we repeated and extended the studies with the epidermal drivers using a higher resolution imaging platform and longer wavelength stimulation (all studies other than Fig. 1), and (3) we observed qualitatively similar results when we repeated stimulation with all drivers using 561 nm light.

We agree that the LED intensity is an important detail of the experimental paradigm. We updated the methods to include intensity measurements for the stimuli used throughout the

manuscript. We also include the intensities here:

- 30 $\mu\text{W}/\text{mm}^2$ for calcium imaging experiments Fig 3B-E, Fig 4A, Fig 3S1A-D, Fig 4S1A
- 300 $\mu\text{W}/\text{mm}^2$ for behavior studies in Fig 2B-E, Fig 1S6, Fig 2S1, Fig 3E-F, Fig 3S2A-C
- 25 $\mu\text{W}/\text{mm}^2$ for behavior studies in Fig 4E-J
- 1.16 $\mu\text{W}/\text{mm}^2$ for behavior studies in Fig 4K

(3) Lines 150 - 152: Although the authors refer to "a stereotyped behavior sequence" in Fig 2D, there are no data supporting this claim in Fig 2. Rather, the data appear to represent proportions of different types of behavior at each time point, rather than behavior sequences. If the authors wish to claim that the data show stereotyped behavior sequences, they should analyze the data using a different method (e.g., Markov models).

We agree that in the absence of additional analysis we should avoid commenting on stereotypy of behavior sequences; we therefore adjusted the text to reflect the tendency of nociceptive behaviors to precede non-nociceptive behaviors. The raster plots shown in Supplemental Fig. 2A illustrate this point: in larvae exhibiting nociceptive behaviors, these behaviors appear first, followed by backing and frequently freezing. As one quantitative readout of this sequence we show that the latency of rolling (nociceptive) is shorter compared with backing or freezing (non-nociceptive) (Fig. 2F, Fig. S2G).

(4) Figure 3A-E: a cursory glance at the data suggests that the most responsive sensory neurons are C1da, with all sensory neurons activated. However, at the behavioral level, only some sensory neurons are activated. If all sensory were activated by Chrimson, what behavioral phenotypes would the authors expect to see? Would it be the same as epidermal activation?

The Reviewer raises an interesting question, but we intentionally avoid comparing the response properties among sensory neurons because of differences in driver strength. Likewise, extrapolating "activation" at the behavioral level is exceedingly difficult if/when multiple sensory neurons are simultaneously activated. In response to the Reviewer's specific question, when all da neurons are activated simultaneously, larvae largely exhibited hunching rather than rolling (Hwang et al., 2007). We find that epidermal stimulation rarely elicits hunching; instead, epidermal stimulation generally triggers nocifensive behaviors followed by non-nocifensive behaviors such as backing and freezing, suggesting an order or priority in neurons activated by epidermal cells (or different response times). Defining the mechanisms by which epidermal cells communicate with different types of sensory neurons is therefore a top priority for future studies.

(5) Figure 3S2; The behavior phenotypes between Fig. 3E, F and Fig 3S2 seems a slightly different. I suggest adding some comments in different behavior phenotype depending on the different GAL4. Specifically, is there increased freezing in some genotypes (e.g., ppk-LexA or NompC-lexA)? Can you show this without TNT data? Is this a background effect or specific GAL4 phenotype?

We currently do not have the driver-only control for this experiment, but our effector-only control experiment (see Fig. 3S2A) suggests that larvae carrying the AOP-TNT insertion exhibit enhanced nociceptive behavioral responses. This point is addressed in our manuscript by the following (copied from the figure legend):

"We note that although baseline rolling probability is elevated in all genetic backgrounds containing the AOP-LexA-TnT insertion, silencing C4da and C3da neurons significantly attenuates responses to epidermal stimulation."

(6) Calcium-free solution is used in Figure 3. Why do the authors still observe calcium influx? Does this mean that internal calcium stores are released? If so, does the calcium influx represent an action potential? How do the authors focus their LED stimulation to activate epidermal cells and avoid activation of the imaging laser?

The specimens were imaged in calcium-free solution to minimize movement artifacts. However, the CNS is wrapped by glial cells and over short timescales such as those used for the imaging we speculate that extracellular calcium persists in the CNS.

(7) It is unclear when animals begin to crawl after the epidermal cells are mechanically stimulated. How do the authors distinguish between peristaltic crawling and a poke by Orai receptors? Although the in vitro experiments beautifully show radial tensions, it is unclear to what extent A-P axis tension (peristaltic crawling) and radial tension (poke) differ. It might be helpful to explain in the discussion section how epidermal cells are selectively activated.

The Reviewer raises an interesting question about the types and thresholds of forces required to elicit epidermal responses. We cannot eliminate the possibility that peristaltic crawling (or crawling through a 3D substrate) stimulates epidermal cells to a certain degree. Indeed, our results demonstrate a dose-dependent response of *Drosophila* epidermal cells and human keratinocytes to radial stretch. However, we do not have any information about selectivity in response to different stimuli, though we agree that this is an intriguing avenue for future studies. For example, we don't know whether stretch-responsive cells are more or less responsive to poke. But, a salient feature of our studies is the recruitment of greater numbers of responders with increasing stimulus intensity, therefore we added the following statement to the discussion to clarify our model:

“Finally, we find that epidermal cells exhibit a dose-dependent response to radial stretch; we therefore anticipate that the output of epidermal cells is likewise dependent on the stimulus intensity. Hence, rather than a fixed threshold beyond which epidermal cells are selectively activated, we hypothesize that increasing stimulus intensities drive increasing signal outputs to neurons.”

(8) Some Protocols are missing. For example, in Figure 4, many stimulus combinations were used to test behavior. How were stimuli of different modalities applied to the animals? Further details need to be provided in the protocols.

We thank the Reviewer for identifying this oversight. The methods section of our original submission detailed most of the stimulus combinations but omitted the opto + mechano combination (4F). We updated our methods to correct these omissions.

(9) It might be helpful if the authors could provide a sample video for each behavior to clarify how they were each defined.

Our manuscript includes a table with a detailed description of the behaviors (Table S2), and we added two annotated videos that show representative behavioral responses to optogenetic nociceptor or epidermis stimulation.

(10) A supplementary summary table of genotypes might be helpful for the reader.

Experimental genotypes are provided in the figure legends, and a detailed list of all alleles used in the study as well as their source is provided in supplemental table S1.

Reviewer #2 (Public Review):

Summary.

The authors provide compelling evidence that stimulation of epidermal cells in Drosophila larvae results in the stimulation of sensory neurons that evoke a variety of behavioral responses. Further, the authors demonstrate that epidermal cells are inherently mechanoresponsive and implicate a role for store-operated calcium entry (mediated by Stim and Orai) in the communication to sensory neurons.

Strengths.

The study represents a significant advance in our understanding of mechanosensation. Multiple strengths are noted. First, the genetic analyses presented in the paper are thorough with appropriate consideration to potential confounds. Second, behavioral studies are complemented by sophisticated optogenetics and imaging studies. Third, identification of roles for store-operated calcium entry is intriguing. Lastly, conservation of these pathways in vertebrates raise the possibility that the described axis is also functional in vertebrates.

Weaknesses.

The study has a few conceptual weaknesses that are arguably minor. The involvement of store-operated calcium entry implicates ER calcium store release. Whether mechanical stimulation evokes ER calcium release in epidermal cells and how this might come about (e.g., which ER calcium channels, roles for calcium-induced calcium release etc.) remains unaddressed. On a related note, the kinetics of store-operated calcium entry is very distinct from that required for SV release. The link between SOC and epidermal cells-neuron transmission is not reconciled. Finally, it is not clear how optogenetic stimulation of epidermal cells results in the activation of SOC.

(1) The involvement of store-operated calcium entry implicates ER calcium store release. Whether mechanical stimulation evokes ER calcium release in epidermal cells and how this might come about (e.g., which ER calcium channels, roles for calcium-induced calcium release etc.) remains unaddressed.

Our studies suggest that mechanically evoked responses in epidermal cells involve both ER calcium release and store-operated calcium entry. Notably, we show that depletion of ER calcium stores before mechanical stimulation, by treating with thapsigargin, reduces (but does not eliminate) mechanically evoked calcium responses in fly epidermal cells (Fig. 6C-6F). Likewise, fly epidermal cells and human keratinocytes both exhibit mechanically evoked calcium responses in the absence of extracellular calcium (10mM EGTA to chelate all free calcium ions). These data support a model whereby mechanical stimuli trigger calcium release from ER stores and influx. Indeed, several cell types have been shown to display mechanically evoked release of calcium from stores. For example, mechanical stimulation of enteroendocrine cells of the gut epithelium results in both calcium release from ER stores and calcium influx across the plasma membrane (Knutson et al., 2023). Similar to our findings, Knutson et al found that depleting stores decreased mechanically evoked calcium signals by over 70% in these gut epithelial stores. In our revised manuscript we have more clearly emphasized these points.

We agree with the reviewer that deciphering the mechanisms by which mechanical stimuli promote ER calcium release and subsequent store-operated calcium entry is an exciting topic to explore. One potential mechanism is the activation of a mechanosensitive receptor that promotes calcium release from the ER via calcium-induced calcium release or IP3 production, as has been proposed for enteroendocrine cells. A recent paper demonstrated that the ER

itself is mechanosensitive and that mechanical stimuli promotes calcium release via the opening of calcium-permeable ion channels in the ER membrane (Song et al., 2024). Determining the relative contributions of store-operated calcium entry and ER calcium release and deciphering their underlying mechanisms will require a thorough investigation of ER calcium channels and receptors, thus we believe this would be beyond the scope of the present manuscript and merits publication on its own. However, we now include this in our discussion as an exciting new direction we aim to pursue.

(2) The kinetics of store-operated calcium entry is very distinct from that required for SV release. The link between SOC and epidermal cells-neuron transmission is not reconciled.

The Reviewer raises an interesting point regarding the mode of epidermal cell-neuronal communication. We demonstrated a requirement for dynamin-dependent vesicle release from epidermal cells in mechanical sensitization. However, the nature of the vesicular pool, the mode and kinetics of release, and the type of neuromodulator released remain to be characterized. Hence, it's not clear that kinetics of synaptic vesicle release is an appropriate comparison. Our studies do demonstrate that behavioral responses to optogenetic epidermal stimulation are relatively slow – on the order of seconds – which is not incompatible with the kinetics of store-operated calcium entry. Furthermore, the primary functional output we define for epidermal mechanosensory responses, mechanical nociceptive sensitization, is apparent 10 sec following the stimulus and persists for minutes in our behavior assays. Consistent with this model, studies of the mammalian touch dome have shown that touch-sensitive Merkel cells secrete neurotransmitters to modulate neurons and promote sustained action potential firing on a similar timescale. Likewise, mechanically evoked ER calcium-release promotes sustained secretion of serotonin from enterochromaffin cells.

(3) It is not clear how optogenetic stimulation of epidermal cells results in the activation of SOC.

We appreciate the opportunity to clarify our results. We demonstrate that optogenetic epidermal stimulation elicits behavioral responses in larvae and calcium responses in somatosensory neurons, but we do not claim that optogenetic epidermal stimulation elicits SOC. Our optogenetic studies demonstrate the capacity for epidermal stimulation to modulate somatosensory function, but we characterize contributions of SOC only to mechanical stimuli which are more physiologically relevant. However, it is worth noting that CsChrimson is a calcium-permeable channel, suggesting that an increase in intracellular calcium may trigger epidermal-evoked neuronal responses and behaviors during optogenetic stimulation.

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