

Phospholipids that Contain Polyunsaturated Fatty Acids Enhance Neuronal Cell Mechanics and Touch Sensation

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SUMMARY

Mechanoelectrical transduction (MeT) channels embedded in neuronal cell membranes are essential for touch and proprioception. Little is understood about the interplay between native MeT channels and membrane phospholipids, in part because few techniques are available for altering plasma membrane composition in vivo. Here, we leverage genetic dissection, chemical complementation, and optogenetics to establish that arachidonic acid (AA), an omega-6 polyunsaturated fatty acid, enhances touch sensation and mechanoelectrical transduction activity while incorporated into membrane phospholipids in *C. elegans* touch receptor neurons (TRNs). Because dynamic force spectroscopy reveals that AA modulates the mechanical properties of TRN plasma membranes, we propose that this polyunsaturated fatty acid (PUFA) is needed for MeT channel activity. These findings establish that polyunsaturated phospholipids are crucial determinants of both the biochemistry and mechanics of mechanoreceptor neurons and reinforce the idea that sensory mechanotransduction in animals relies on a cellular machine composed of both proteins and membrane lipids.

INTRODUCTION

The sensory neurons embedded in our skin use mechanoelectrical transduction (MeT) channels to detect mechanical stimuli delivered by a feather's brush, a pin's prick, or a mobile phone's buzz. Analogous sensory neurons in joints and muscles help to maintain balance and posture, whereas others innervate the aorta and regulate heart rate on a beat-by-beat basis. Most, if not all, MeT channels belong to macromolecular complexes whose protein constituents have been identified only for a few mechanoreceptor cells. In *C. elegans*, it has been shown that members of the degenerin/epithelial sodium channel (DEG/

ENaC) (MEC-4, MEC-10, and DEG-1) and transient receptor potential (TRP) channel (TRP-4, also known as CeNOMPC) families are pore-forming subunits of MeT channels in vivo (Arnadóttir et al., 2011; Geffeney et al., 2011; Kang et al., 2010; O'Hagan et al., 2005). Additional MeT channel subunits have been identified in fruit flies and mice: NOMPC is thought to be a pore-forming subunit in fly mechanoreceptor neurons (Gong et al., 2013; Yan et al., 2013; Zhang et al., 2013); piezo proteins are thought to function as MeT channels in body mechanoreceptor neurons and in red blood cells (Bae et al., 2013; Coste et al., 2010; Kim et al., 2012); TMHS and TMC proteins are required for mechanotransduction by inner-ear hair cells in mice (Pan et al., 2013; Xiong et al., 2012). Thus, whereas many of the proteins needed to form MeT channels in mechanoreceptor cells have been identified, very little is known about how the composition of the membrane modulates MeT channel function.

One of the many advantages of studying touch sensitivity in *C. elegans* touch receptor neurons (TRNs) is that they generate electrical signals in response to cuticle deflection and transmit signals mainly via electrical synapses onto interneurons that control locomotion (Chalfie and Sulston, 1981; Chalfie et al., 1985; Goodman, 2006). At least five genes encode membrane proteins required to form the MeT channel complex in the TRNs. Four are *mec* (mechanosensory abnormal) genes that can mutate to disrupt gentle touch sensation, and mutations in the fifth disrupt both touch sensation and locomotion (Chalfie and Sulston, 1981; Zhang et al., 2004): *mec-4*, *mec-10*, *mec-2*, *mec-6*, and *unc-24*. These genes encode two DEG/ENaC ion channel subunits (MEC-4 and MEC-10), two stomatin homologs (MEC-2 and UNC-24), and a paraoxonase-like protein (MEC-6) (Arnadóttir and Chalfie, 2010). Both MEC-2 and MEC-6 are thought to interact with cholesterol (Brown et al., 2008; Chelur et al., 2002; Huber et al., 2006). Indeed, MEC-2 binds cholesterol, and its function as an auxiliary channel subunit depends on this ability (Brown et al., 2008; Huber et al., 2006). Therefore, it is likely that membrane composition plays an important role in modulating the response of TRNs to mechanical stimuli.

When incorporated into phospholipids, polyunsaturated fatty acids (PUFAs) confer fluidity and flexibility to membranes (Rawicz et al., 2000; Rajamoorthi et al., 2005); consequently, they could modulate membrane protein function. Free PUFAs have been shown to modulate voltage-gated and mechanosensitive

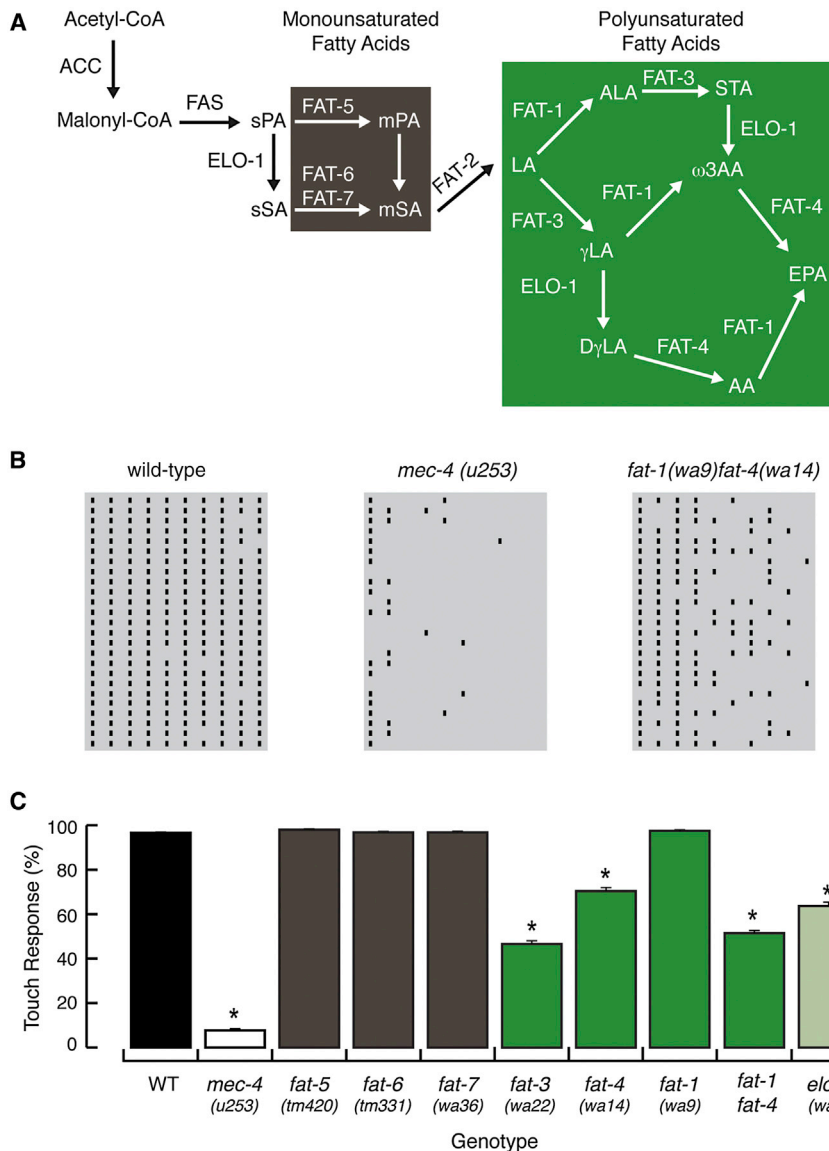


Figure 1. Arachidonic and Eicosapentaenoic Acids Are Required for Touch Sensitivity

(A) Fatty acid synthesis in *C. elegans*, adapted from Watts (2009). Brown and green boxes denote the enzymatic cascade needed to synthesize mono-unsaturated and polyunsaturated fatty acids, respectively. AA, arachidonic acid (20:4n-6); ALA, α -linolenic acid (18:3n-3); D γ LA, dihomo-linolenic acid (20:3n-6); EPA, eicosapentaenoic acid (20:5n-3); FAS, fatty acid synthase; LA, linolenic acid (18:2n-6); STA, stearidonic acid (18:4n-3); γ LA, γ -linolenic acid (18:3n-6); ω 3AA, ω -3 AA (20:4n-3). (B) Raster plots displaying the response to gentle touch of cohorts of 25 worms. Bars indicate trials that elicited reversals or pauses. Columns and rows represent trials and worms, respectively. (C) Touch response in wild-type (WT), *mec-4*, and *fat* mutants. At least 75 animals were tested blind to genotype. Bars are mean \pm SEM. The asterisk indicates values significantly different from WT; Kruskal-Wallis and Dunn's multiple comparisons tests; $p < 0.001$. See also Figure S1.

TRN-dependent behavioral responses, thereby identifying membrane phospholipids containing AA as critical for touch sensitivity. Arachidonic acid is likely synthesized within TRNs in vivo, because we show that enzymes needed for its synthesis are expressed in TRNs. We used an optogenetic approach to show that the defect in touch sensation likely reflects a loss of mechanotransduction rather than lack of excitability or downstream signaling. Finally, we found that the membrane viscoelastic properties of TRNs lacking C20 PUFAs are altered (i.e., membrane bending and viscosity), yielding less flexible membranes than wild-type, as determined by atomic force

ion channels in heterologous cells (Balleza et al., 2010; Kim, 1992; Maingret et al., 2000). Genetic dissection in *C. elegans* shows that sensory and motor neurons depend on PUFAs for their function (Kahn-Kirby et al., 2004; Lesa et al., 2003). However, the physiological role of this modulation remains to be determined. Unlike mammals, worms can synthesize PUFAs de novo from acetyl-coenzyme A using a series of fatty acid desaturase and elongase enzymes, known as FAT and ELO proteins, respectively (Wallis et al., 2002; Watts and Browse, 2002). Mutants defective in *fat* and *elo* genes have altered PUFA content (Watts and Browse, 2002) and grow into normal adults with mild phenotypes, except for *fat-2* mutants. We exploited this knowledge to ask whether PUFAs are needed for touch sensation and the normal function of the *C. elegans* TRNs.

Here, we demonstrate that disrupting arachidonic acid (AA) content or its incorporation into phospholipids impairs

microscopy (AFM)-based single-tether extrusion. Our findings demonstrate that, when AA is part of membrane phospholipids, it modulates the mechanical properties of *C. elegans* TRN membranes and is crucial for touch sensation.

RESULTS

Arachidonic Acid Is Required for Touch Sensation

We used a behavioral test to investigate the contribution of lipids to touch sensitivity, exploiting the well-characterized enzymatic cascade (Figure 1A) that leads to the production of C20 PUFAs in *C. elegans* (Watts, 2009; Watts and Browse, 2002). Touch sensitivity was measured with a ten-trial touch assay that consisted of stroking an eyebrow hair across the body of young adults, alternating between the anterior and posterior part of the worm (Hart, 2006). Trials that elicited a pause or avoidance

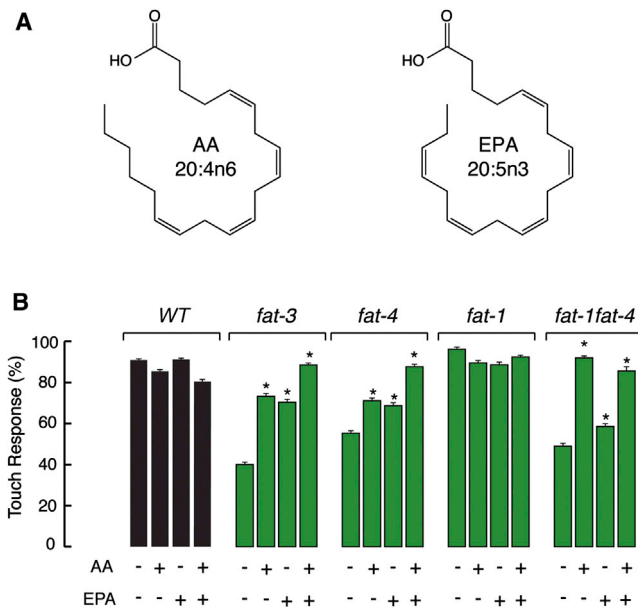


Figure 2. Arachidonic Acid Rescues Touch Sensitivity

(A) Chemical structures of arachidonic (AA) and eicosapentaenoic acids (EPA). (B) Touch response in *fat* mutants grown on NGM plates containing C20 PUFAs AA and EPA. NGM was supplemented with PUFAs as previously described (Watts et al., 2003). At least 75 animals were tested blind to genotype and treatment. Bars are mean \pm SEM. The asterisk indicates values significantly different from experimental control; Kruskal-Wallis and Dunn's multiple comparisons tests; $p < 0.001$.

were considered positive. Figure 1B shows raster plots for wild-type worms, *mec-4(u253)* mutants that lack functional MeT channels, and for *fat-1(wa9)fat-4(wa14)* double mutants, whereas Figure 1C shows the average number of positive trials (of ten) as a function of genotype. We used this ten-trial touch assay because it allowed us to identify modest defects that could not have been detected in three-trial touch assays used previously (Kahn-Kirby et al., 2004). This is because loss of touch-evoked avoidance was evident only after the third stimulus (Figures 1B and 1C). The defects found in *fat* mutants differ from the habituation evident in wild-type worms after nine or ten trials (Hart, 2006).

With this survey, we identified four mutants with moderate, but reproducible, defects in touch sensation (Figure 1C): *elo-1*, *fat-3*, *fat-4*, and *fat-1fat-4*. ELO-1 is required to elongate fatty acids and has a critical role in the synthesis of C20 PUFAs (Figure 1A). Its touch impairment suggests that C20 PUFAs may be critical for normal touch sensitivity (Figure 1C). Consistent with this idea, *fat-3* mutants, which lack the desaturase activity required to synthesize both C18 and C20 PUFAs (Figure 1A, green box), are also partially touch insensitive. However, *fat-3* mutants also have developmental and neuromuscular junction defects (Lesa et al., 2003; Watts et al., 2003) that complicate interpretation. As a result, *fat-3* mutants were not analyzed further.

The *fat-4* gene also encodes a desaturase. FAT-4 acts on C20 PUFAs to produce AA and eicosapentaenoic acid (EPA), the largest PUFAs in *C. elegans* (green box, Figures 1A and 2A) and, as a result, *fat-4* mutants lack AA and EPA. They also

contain 2-fold more of the upstream fatty acid, ω -3 AA, than wild-type (Kahn-Kirby et al., 2004). As shown in Figure 1C, the touch response of *fat-4* mutants is approximately one-third that of wild-type worms. This decrease in touch sensitivity could be due to the loss of AA and EPA or to the accumulation of ω -3 AA. A central role for ω -3 fatty acids (alpha-linolenic acid [ALA], stearidonic acid [STA], ω -3AA, and EPA) seems unlikely, however, because *fat-1* mutants cannot synthesize these fatty acids (green box, Figure 1A) but have wild-type touch sensitivity (Figure 1C). However, *fat-1* has approximately 20 times more AA than wild-type (Kahn-Kirby et al., 2004), which could potentially compensate for the loss of ω -3 fatty acids. The *fat-1fat-4* double mutant has no gross defects in development or locomotion and lacks the ability to synthesize both AA and ω -3 fatty acids, including EPA (Kahn-Kirby et al., 2004), providing an additional tool for testing whether or not AA is essential. Indeed, the touch response of *fat-1fat-4* double mutants was half that of wild-type (Figure 1C). Collectively, these results establish that mutants lacking C20 PUFAs have defects in touch sensation and implicate AA as a key fatty acid required for normal touch sensation.

As with any genetic dissection of an enzymatic cascade, however, loss of desaturases and elongases results not only in the loss of downstream products, but also significant changes in the levels of upstream substrates (Kahn-Kirby et al., 2004). Because this complicates interpretation, we sought other ways to manipulate fatty-acid content and settled on growth temperature and chemical complementation of *fat* mutants as effective methods. Prior work has shown that worms grown at 15°C have less AA, more EPA, and more of the upstream substrate, linolenic acid, compared to worms grown at 20°C or 25°C (Tanaka et al., 1996). When we tested the effect of growth temperature on touch sensitivity in wild-type worms, however, we found no significant effect. In contrast, *fat-1fat-4* mutants grown at 15°C were significantly less touch sensitive than those grown at 20°C or 25°C (Figure S1). Thus, wild-type worms, but not *fat-1fat-4* mutants, maintain constant touch sensitivity across a 10-degree range in growth temperature. This result suggests that the ability to synthesize C20 PUFAs is needed for touch sensation and for proper thermal homeostasis of this behavior.

Next, we used chemical complementation of *fat-1*, *fat-4*, and *fat-1fat-4* mutants to learn more about the role of AA and EPA in touch sensation. Supplementing nematode growth media (NGM) with AA, EPA, or both leads worms to incorporate these fatty acids through their diet (Watts and Browse, 2002). This allowed us to differentiate between the absence of C20 PUFAs (AA and EPA), the accumulation of upstream fatty acids, or developmental defects. Wild-type and *fat-1* worms did not change their ability to respond to touch when NGM was supplemented with AA or EPA, suggesting that an excess of PUFAs does not affect touch sensitivity (Figure 2A). Complementation of *fat-3* and *fat-4* mutants with AA or EPA improved their ability to respond to touch compared to the negative control (Figure 2B); however, only when AA and EPA were supplemented together were these worms able to respond like wild-type. Taken together, these results provide additional evidence that it is the absence of AA and EPA that causes the touch defect observed in *fat-3* and *fat-4* worms, rather than the accumulation of upstream precursors. The double mutant *fat-1fat-4*, unlike the

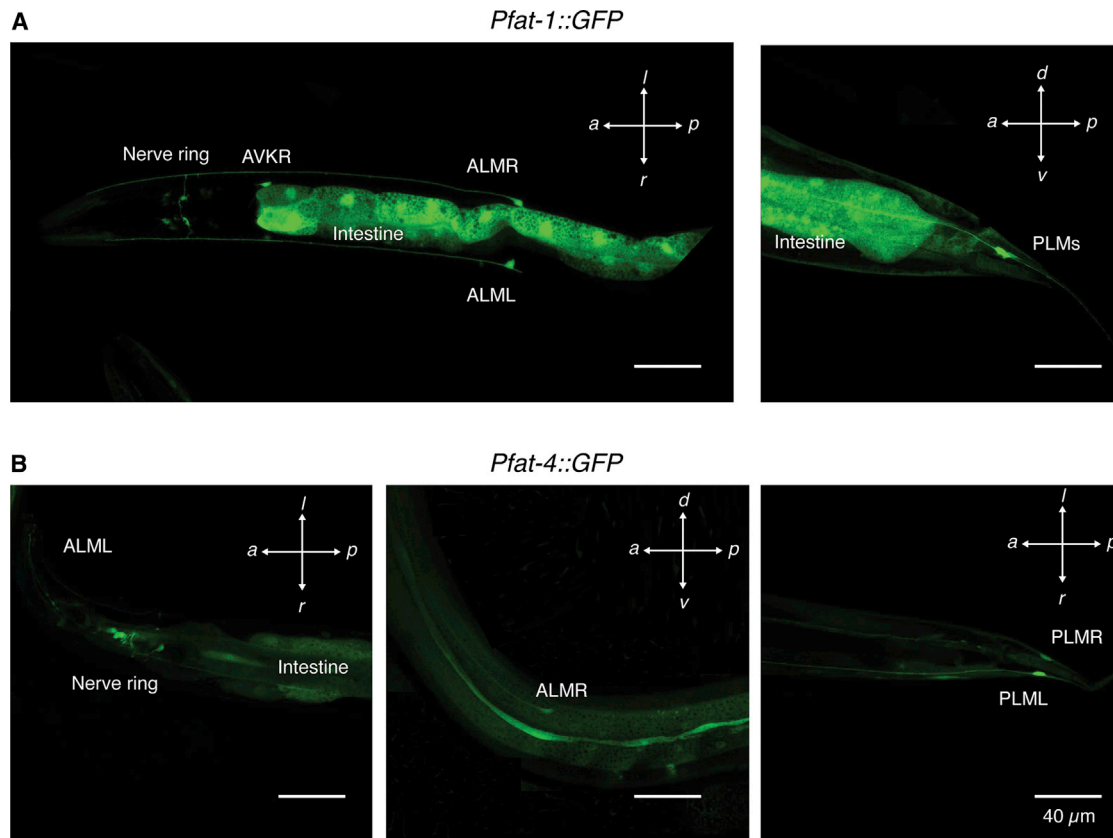


Figure 3. Touch Receptor Neurons Express *fat-1* and *fat-4*

(A and B) Worms express GFP under the control of promoters for *fat-1* (A) and *fat-4* (B) in many cells including the TRNs: ALM (L/R), PLM (L/R), and the nerve ring. The scale bars represent 40 μm in all panels.

fat-4 single mutant, does not transform AA into EPA, even when exogenous AA is provided (green box, Figure 1A). We found that AA, but not EPA, could recapitulate wild-type behavior in *fat-1fat-4* double mutants (Figure 2B). These chemical complementation assays demonstrated that AA, but not EPA, is a crucial PUFA for the modulation of touch sensitivity.

TRNs Coexpress *fat-1* and *fat-4*, which Are Dispensable for TRN Shape and MeT Channel Distribution, but Not for TRN Function

To determine whether C20 PUFAs are likely to be present in TRNs, we built fusion constructs to drive the expression of GFP from *fat* promoters. These experiments revealed that *fat-1* and *fat-4* are expressed in TRNs through larval development into adulthood (Figure 3), suggesting that AA and EPA are continuously synthesized by TRNs in situ. Similar expression patterns were observed in at least three independent transgenic lines. To test for potential defects in neuron morphology arising during development that could contribute to the poor touch sensitivity of worms lacking C20 PUFAs, we crossed *fat* mutants with transgenic *uls31* animals that express GFP exclusively in the TRNs (O'Hagan et al., 2005). Young adult worms that lack C20 PUFAs display GFP-labeled TRNs (Figure S2) with morphological features similar to wild-type (i.e., cell-body position, neurite exten-

sion and position along the body, and branching). The TRN morphologies of *elo-1*, *fat-4*, and *fat-1fat-4* worms were also comparable to that of wild-type (data not shown). We also found that the pore-forming subunit of the MeT channel complex (MEC-4::yellow fluorescent protein [YFP]) displays a normal punctate distribution (Cueva et al., 2007) in TRNs lacking C20 PUFAs (Figure S3). Therefore, we conclude that the impaired touch response of mutants lacking C20 PUFAs does not arise from gross defects in TRN development, morphology, or from mislocalization of the MeT channel complex.

Having established that, whereas *fat-1fat-4* mutants are partially touch insensitive, they have normal TRN morphology and distribution of MEC-4-dependent MeT channels, we next sought to determine the locus of the defect in the signaling pathway linking touch to behavior (Figure 4A). In principle, *fat-1fat-4* mutants could have a defect in mechano-electrical transduction, TRN depolarization, synaptic transmission, interneuron or motor neuron function, muscle activity, or a combination of these factors. To do this, we crossed *fat* mutants with transgenic worms expressing channelrhodopsin (ChR2) exclusively in the TRNs. Exogenous supplementation with all-*trans*-retinal (ATR) is required for ChR2-dependent blue-light stimulation in *C. elegans* (Nagel et al., 2005), which provides a simple way to control for nonspecific effects due to optical stimulation

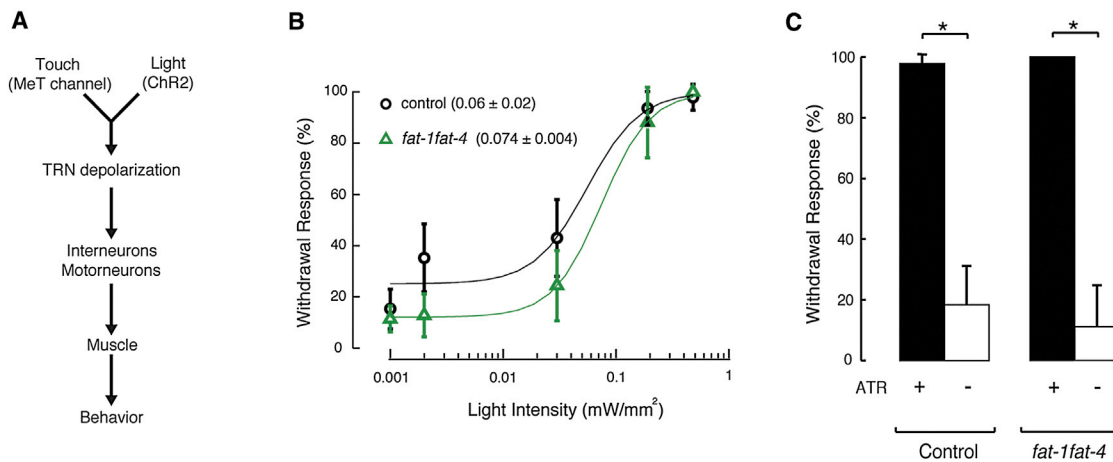


Figure 4. C20 PUFA-Deficient Mutants Respond Normally to Optogenetic Stimulation

(A) Schematic of the circuitry and motor neuron program downstream of TRNs.

(B) Withdrawal response elicited by illuminating young adult control and *fat-1fat-4* worms expressing ChR2 exclusively in their TRNs with a 470 nm light for 1 s every 30 s at these irradiance values (0.001, 0.002, 0.03, 0.19, and 0.48 mW/mm²) in the presence of all-*trans*-retinal. A dose-response curve was fitted to the data. At least ten animals were tested blind to genotype and treatment. Circles and triangles are mean ± SD.

(C) Withdrawal response elicited by illuminating young adult worms (control and *fat-1fat-4* mutants) expressing ChR2 exclusively in their TRNs with a 470 nm light for 1 s every 30 s at 0.48 mW/mm² irradiance in the presence or absence of all-*trans*-retinal. At least ten animals were tested blind to genotype and treatment. Bars are mean ± SEM. The asterisk indicates values significantly different from experimental control; Kruskal-Wallis and Dunn's multiple comparisons tests; $p < 0.001$.

and transgene expression. In this framework, mutants with defects downstream of mechanoelectrical transduction will exhibit defects in both touch and light-evoked behaviors, whereas mutants with defects in mechanoelectrical transduction will behave like wild-type worms when stimulated with blue light. Accordingly, we tested the response of young adults in the presence of ATR to flashes of blue light at different intensities, thus triggering withdrawal responses in the control and *fat-1fat-4* worms (Figure 4B). We found that the midpoints of these curves were similar for control (0.06 ± 0.02 mW/mm²; mean ± SD) and *fat-1fat-4* (0.074 ± 0.004 mW/mm²) worms. Figure 4C shows the results after delivering flashes at saturating light intensity (0.48 mW/mm²). This approach allowed us to compare responses to blue light and to touches delivered by the classical method (Hart, 2006), which delivers forces of 100 μN or more, as estimated by stroking a calibrated piezoresistive cantilever with an eyebrow hair (E.A. Mazzochette, K.-W. Jung, M.B.G, and B.L. Pruitt, unpublished data), whereas behavioral responses to touch saturate near 1 μN (Petzold et al., 2013). Consistent with the idea that TRN excitability and downstream signaling are intact, we found that *fat-1fat-4* mutants respond like control worms (Figures 4B and 4C).

With this optogenetic approach, we cannot exclude the possibility that loss of C20 PUFAs affects other ion channels that may be present in the TRNs. For example, certain potassium channels are known to be potentiated by free AA (Callejo et al., 2013; Hao et al., 2013). In principle, such an effect could compensate for a partial loss of MeT channel function at saturating light intensities. Such an effect is unlikely because it would shift the light-response curve toward lower-light intensities or increase the steepness of the curve. We found no evidence for either effect in *fat-1fat-4* mutants. Thus, these findings imply that worms lacking C20 PUFAs have a specific defect in mecha-

noelectrical transduction, rather than general defects in TRN excitability; synaptic transmission; or in the function of downstream interneurons, motor neurons, or muscles.

Arachidonic-Acid-Containing Phospholipids Modulate Touch Sensation

Our findings implicate AA as a key regulator of TRN mechanotransduction but do not distinguish between effects of AA itself or its metabolites, such as the endocannabinoids anandamide (AEA) and 2-arachidonyl glycerol (2-AG). Recent studies show that both AEA and 2-AG are present in wild-type worms but absent in *fat-3* and *fat-4* mutants (Lehtonen et al., 2011; Lucanic et al., 2011). Thus, it is possible that the touch-response defect in these mutants is due to the lack of AA, AEA, or 2-AG. To identify the mechanism by which AA modulates touch sensation, we supplemented NGM with AEA, 2-AG, and eicosatetraenoic acid (ETYA), a structural analog of AA that cannot be metabolized (Figure 5A). As shown in Figure 5B, complementing with AEA or 2-AG did not reconstitute wild-type touch sensitivity. By contrast, ETYA is able to rescue the touch defect in *fat-1fat-4* mutant comparable to AA (Figure 5C). Up to this point, our data support a model where AA could exert its effect as a free fatty acid or as a constituent of membrane lipids, but not as a precursor of its metabolites.

Given that AA can be incorporated into membrane phospholipids (Figure 6A), we investigated whether AA-containing phospholipids regulate the response to mechanical stimuli. *C. elegans* has two conserved lysophospholipid transferase enzymes responsible for incorporating C20 PUFAs into the sn2 position of phospholipids: MBOA-7 links AA and EPA to phosphatidylinositol (PI) (Lee et al., 2008) and MBOA-6 links AA to phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) (Matsuda et al., 2008). If AA acts

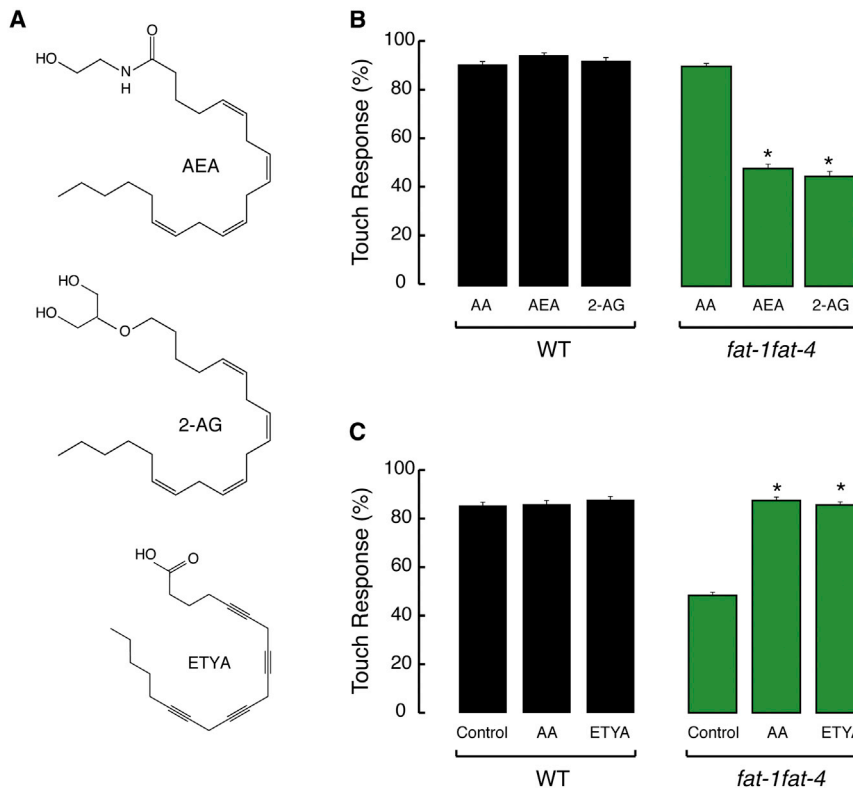


Figure 5. Arachidonic Acid, but Not Its Metabolites, Are Needed for Touch Sensitivity

(A) Chemical structures of arachidonoyl ethanamide (AEA), 2-arachidonoyl glycerol ether (2-AG), and eicosatetraenoic acid (ETYA).

(B) Touch response in wild-type (WT) and *fat-1fat-4* animals grown in the presence of AA and its endocannabinoid metabolites AEA and 2-AG. At least 75 animals were tested blind to genotype and treatment. Bars are mean \pm SEM. The asterisk indicates values significantly different from experimental control; Kruskal-Wallis and Dunn's multiple comparisons tests; $p < 0.001$.

(C) Touch response in wild-type (WT) and *fat-1fat-4* animals grown in the presence of AA and its nonmetabolizable analog ETYA. At least 75 animals were tested blind to genotype and treatment. Bars are mean \pm SEM. The asterisk indicates values significantly different from experimental control; Kruskal-Wallis and Dunn's multiple comparisons tests; $p < 0.001$.

while incorporated into membrane lipids, then mutants deficient in the enzymes MBOA-7 and MBOA-6 could be deficient in TRN-mediated behavior. *mboa-7(gk399)* mutants have wild-type touch sensitivity, suggesting that AA-containing PI is not necessary for touch sensitivity (Figure 6B). Unfortunately, *mboa-6*-null mutants are embryonic lethal (Matsuda et al., 2008). We circumvented this severe phenotype by using RNAi to knock down *mboa-6* postembryonically (*mboa-6* knockdown worms have normal locomotion). Knocking down the expression of *mboa-6* in wild-type worms resulted in animals fully sensitive to touch (Figure 6C), consistent with prior reports that neurons are resistant to RNAi in wild-type worms (Kamath et al., 2001; Timmons et al., 2001). To knock down the expression of *mboa-6* in neurons, we used a recently engineered transgenic strain (*Punc-119::SID-1*) that increases RNAi sensitivity in neurons through expression of the SID-1 transmembrane protein (Calixto et al., 2010). Following this strategy, we found that knocking down *mboa-6* decreased touch sensitivity by $\sim 30\%$, which is $\sim 20\%$ more sensitive than knocking down the pore-forming subunit MEC-4 (Figure 6C). These results suggest that full expression of *mboa-6* is required to achieve wild-type touch sensitivity.

To determine whether *mboa-6* or the phospholipids it helps to synthesize are responsible for modulating touch sensitivity, we combined RNAi-mediated gene knockdown and chemical complementation. First, we determined that supplementing NGM with phospholipids did not interfere with RNAi knockdown. To this end, we knocked down *mec-4* in the presence of phospholipids loaded with stearic acid (18:0) and arachidonic acid (20:4) loaded at their *sn1* and *sn2* positions, respectively (Fig-

ure S4): (18:0)(20:4)PC, (18:0)(20:4)PE, and (18:0)(20:4)PS. This manipulation had no detectable effect on touch sensitivity, indicating that phospholipid supplementation was compatible with RNAi knockdown. Next, we sought to restore wild-type behavior in *mboa-6(mai)* worms when complemented with (18:0)(20:4)PC, (18:0)(20:4)PE, and (18:0)(20:4)PS in combination (Figure 6D, left). Supplementing with (18:0)(20:4)PE, and (18:0)(20:4)PS, but not (18:0)(20:4)PC, restored touch sensitivity to nearly wild-type levels in worms treated with *mboa-6(mai)* (Figure 6D, right). The ability of (18:0)(20:4)PE and (18:0)(20:4)PS, but not (18:0)(20:4)PC, to rescue *mboa-6(mai)*-induced touch defects could reflect the preferential localization of PS and PE phospholipids to the inner leaflet of the cell membranes. (PC lipids localize primarily to the outer leaflet of the plasma membrane [Fadeel and Xue, 2009; Leventis and Grinstein, 2010]). Collectively, these results suggest that membranes with AA-containing PE and PS modulate mechano-electrical transduction in living worms.

C20 PUFAs Regulate Membrane Mechanics in Touch Receptor Neurons

Based on the finding that C20 PUFAs modulate touch sensitivity while incorporated in membrane phospholipids, we hypothesized that simultaneous loss of *fat-1* and *fat-4* function and, therefore, C20 PUFAs would alter the mechanical properties of TRN membranes. To test this idea directly, we isolated cells from *uls31* control and *fat-1fat-4;uls31* embryos expressing exclusively GFP in the TRNs, grew them in culture for ≥ 24 hr, and used an AFM to pull single lipid membrane nanotubes or tethers (Figure 7A, inset). To further characterize membrane mechanics, we measured tether force as a function of pulling rate and fit the resulting force-velocity curves according to a biophysical model of the relationship between the dynamics of extrusion and the mechanical properties of the membrane (Borghi and Brochard-Wyart,

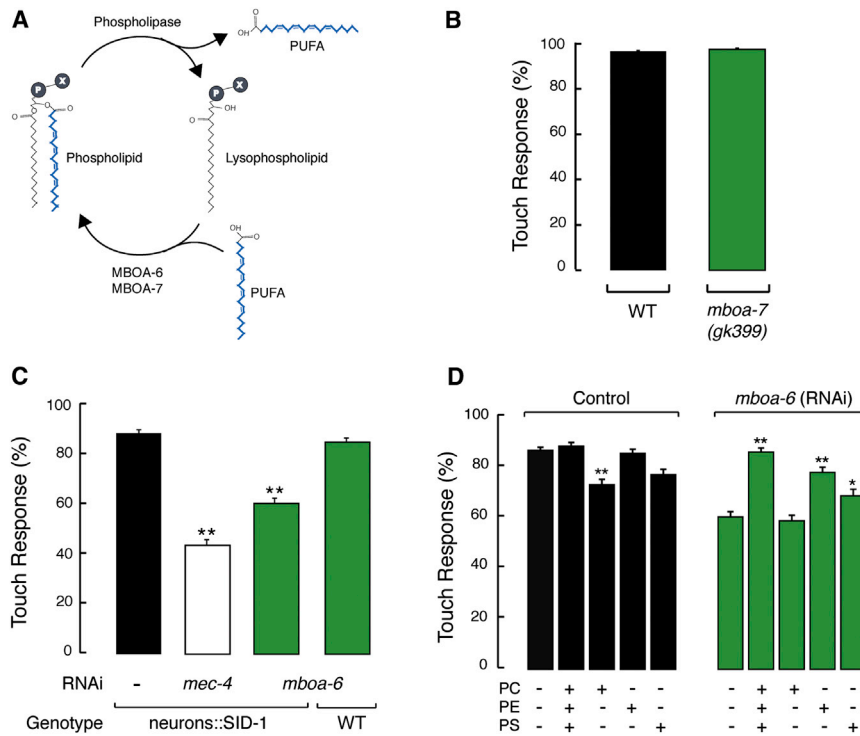


Figure 6. Arachidonic-Acid-Containing Membrane Phospholipids Modulate Touch Sensitivity

(A) Schematic representation of the phospholipid synthesis cycle, where MBOA-7 and MBOA-6 (lysophospholipid acyltransferases) act to incorporate PUFAs into membrane phospholipids (Matsuda et al., 2008) and phospholipase releases lysophospholipids and free PUFAs. Adapted from Hishikawa et al. (2008).

(B) Touch response in *mboa-7* mutants. At least 75 animals were tested blind to genotype. Bars are mean \pm SEM.

(C) Touch response after knocking down the expression of *mboa-6* and *mec-4* with RNAi, in wild-type (WT) and transgenic worms that express *sid-1* exclusively in neurons (neurons::SID-1). At least 75 animals were tested blind to genotype and treatment. Bars are mean \pm SEM. Double asterisks indicate values significantly different from experimental control; Kruskal-Wallis and Dunn's multiple comparisons tests; $p < 0.001$. See also Figure S4.

(D) Touch response after knocking down *mboa-6* in transgenic worms that express *sid-1* exclusively in neurons and after supplementing NGM with the AA-containing phospholipids. PC, (18:0)(20:4) phosphatidylcholine; PE, (18:0)(20:4) phosphatidylethanolamine; PS, (18:0)(20:4) phosphatidylserine. At least 75 animals were tested blind to genotype. Bars are mean \pm SEM. The asterisk indicates values significantly different from experimental control; Kruskal-Wallis and Dunn's multiple comparisons tests; ** $p < 0.001$ and * $p < 0.05$.

2007; Brochard-Wyart et al., 2006). This procedure allowed us to extrapolate the static tether force at zero velocity (F_0 , the intersection between the fit and the y axis) and to assess changes in effective membrane viscosity, bending stiffness, or the density of membrane-cytoskeleton binders (Diz-Muñoz et al., 2010). As shown in Figure 7A, the curves overlap at low velocity ($p = 0.24$ at $1 \mu\text{m/s}$; Student's t test) and diverge as velocity increases such that larger forces are generated when pulling tethers from *fat-1fat-4* mutant TRNs than from control neurons. Thus, whereas static force (F_0) is similar in control ($20 \pm 3 \text{ pN}$; mean \pm SEM) and *fat-1fat-4* mutant ($23 \pm 8 \text{ pN}$) TRNs, the dynamics of tether extrusion are dramatically altered by the loss of C20 PUFAs ($p < 0.05$). The divergence observed at high velocities could reflect differences in membrane bending stiffness, effective viscosity, the density of binders between the membrane and the cytoskeleton, or a combination of all three factors (Borghi and Brochard-Wyart, 2007; Brochard-Wyart et al., 2006). Because the composition of the cortical cytoskeleton is likely to be similar in control and *fat-1fat-4* TRNs, we propose that the differences come mainly from a change in membrane bending stiffness and effective viscosity. Collectively, these results indicate that TRN membranes lacking C20 PUFAs have different mechanical properties and are likely to be less flexible than control TRN membranes.

DISCUSSION

Classical genetics has uncovered many of the proteins crucial to form MeT channels in *C. elegans* TRNs and other mechanore-

ceptor cells (Arnadóttir and Chalfie, 2010), including mammalian auditory and vestibular hair cells (Pan et al., 2013; Xiong et al., 2012). This approach lacks the power to uncover roles for specific lipids, however, because a plethora of genes are needed for lipid synthesis and metabolism. To circumvent this difficulty, we exploited the well-characterized enzymatic cascade used by *C. elegans* to synthesize fatty acids, including C20 PUFAs (Watts, 2009). By surveying how mutants lacking mono- and polyunsaturated fatty acids (*fat* and *elo* mutants) respond to gentle touch and by using chemical complementation of selected *fat* mutants, we established that AA is needed for touch sensation. Rather than acting as a signaling molecule or as a metabolic precursor for other signaling molecules, AA exerts its effect on touch sensation when it is incorporated into phospholipids (Figure 6).

Though highly penetrant, the touch defect seen in *fat* mutants is less severe than the defect present in *mec-4*-null mutants that lack MEC-4, an indispensable pore-forming subunit of the MeT channel complex (O'Hagan et al., 2005). Also, *fat* mutants responded to initial, but not later, trials in the ten-trial touch assay (Figure 1B). Though the basis for this adaptation is not yet clear, it differs from the habituation seen in wild-type animals, which requires more stimulation, is less severe, and depends primarily on the neural circuitry linking the TRNs to motor output (reviewed by Ardiel and Rankin [2008]). Both of these features of *fat* mutant touch sensation point toward flaws in the function of MeT channels, but not in their expression or distribution. Indeed, the distribution of MEC-4::YFP puncta was indistinguishable in *fat-1fat-4* mutant and control TRNs (Figure S3).

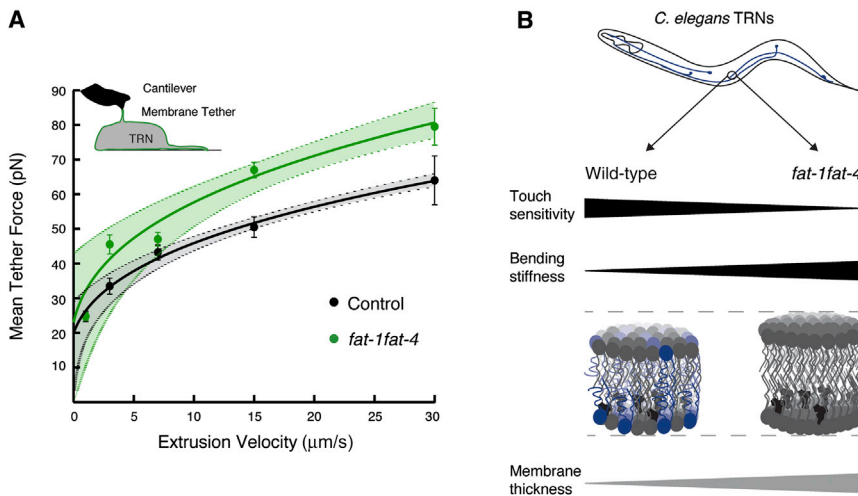


Figure 7. C20 PUFAs Contribute to Membrane Mechanics in Touch Receptor Neurons

(A) AFM force spectroscopy on isolated TRNs expressing GFP. Plot of tether force versus cantilever retraction speed. Velocities between 1 and 30 $\mu\text{m/s}$ were tested. The equation $f^3 - fF_0^2 = av$ was fitted to the data, where f is the tether force at velocity v , F_0 is the static force, and a is the coefficient characterizing the dynamics of extrusion (Borghi and Brochard-Wyart, 2007; Brochard-Wyart et al., 2006). Circles are mean \pm SEM. The dotted lines indicate the 95% confidence bands for the fit. At least 20 TRNs were tested. Inset shows a graphical representation of the tether-extrusion experiment, in which an AFM cantilever was coated with peanut lectin and approached onto an isolated TRN and kept in contact for 0.4 s upon retraction; a lipid tube eventually formed in 20% of all contacts.

(B) Schematic of the differences in behavior and

TRN membrane mechanics in wild-type and *fat-1fat-4* worms. Blue, AA-containing phospholipids; gray, phospholipids with less than four unsaturations; and black, cholesterol. Black wedges are measured quantities; the gray wedge shows a predicted difference in membrane thickness.

At least two lines of additional evidence support the idea that AA acts within the TRNs to ensure proper MeT channel function. First, the TRNs have the capacity to synthesize AA because they express both of the desaturase genes (*fat-1* and *fat-4*) needed for AA synthesis (Figures 1A and 3). Second, transgenic *fat-1fat-4* mutants expressing the light-activated ChR2 protein exclusively in the TRNs have impaired touch sensation, but respond just like control transgenics to flashes of blue light (Figure 4). Thus, most, if not all, signaling events downstream of the activation of MeT channels function normally in AA-deficient *fat-1fat-4* mutants. Whereas we cannot exclude compensatory, pleiotropic effects within the TRNs, the simplest model consistent with all of our data is that loss of AA-containing phospholipids compromises the ability of touch to activate MeT channels.

The biophysical properties of membrane lipids modulate integral membrane proteins, including mechanosensitive channels (Andersen and Koeppel, 2007; Brown, 2012; Phillips et al., 2009). For instance, reconstituting MscL channels in thin phospholipid bilayers containing short acyl chains stabilizes open states, whereas reconstitution in thicker phospholipid bilayers containing longer-chain fatty acids stabilizes closed states (Perozo et al., 2002). Including PUFAs in phospholipid membranes also modulates the physical properties of bilayers, increasing disorder and decreasing thickness (Salmon et al., 1987). Thus, depleting native plasma membranes of PUFAs could increase membrane thickness as well as the energy required for deformation, leading to higher bending rigidity. Experimental evidence collected from synthetic phospholipids supports this idea because PUFA-containing bilayers are more deformable than ones containing saturated fatty acids (Rawicz et al., 2000; Rajamoorathi et al., 2005). Our data on native TRN membranes agree with these biophysical studies: The plasma membrane of *fat-1fat-4* mutant TRNs that lack C20 PUFAs generates the force-velocity curves expected if PUFA deficiency increased membrane bending rigidity. Additionally, *fat-1fat-4* worms cultivated at 15°C are less touch sensitive than those grown at 20°C or 25°C, a

result that suggests that the further decrease in membrane fluidity expected at cooler temperatures exacerbates the touch defect of animals lacking C20 PUFAs. Within this framework, we propose that the response of TRNs to mechanical stimuli is regulated by lipid composition and that the presence of multiple unsaturated bonds creates a distinct environment that enables the function of the MeT channel complex.

Current models of sensory mechano-electrical transduction posit that mechanical energy is delivered to MeT channels either through the plasma membrane or mainly by protein tethers (Kung, 2005; Zanini and Göpfert, 2013). As the plasma membrane is a vital element of both these models, our findings cannot differentiate among them. Instead, we provide evidence that AA-containing phospholipids are required for touch sensation and suggest that these lipids are needed to regulate the sensitivity or time course of MeT channel activation. Though additional studies will be needed to test this model in detail, this study helps to determine the specialized lipid environment essential for MeT channel function.

How might variation in the composition and physical properties of the plasma membrane alter the mechano-electrical transduction process? The picture emerging from our work is that the TRN membranes of wild-type animals include AA-containing phospholipids (PE and PS, which localize primarily to the inner leaflet) and cholesterol, and these elements are balanced to enhance and modulate touch sensitivity (Figure 7B). From previous studies, we know that cholesterol is needed for proper function of the MEC-4-channel complex and it is recruited by the stomatin-like protein MEC-2 (Brown et al., 2008; Huber et al., 2006). Recently, Anishkin and Kung (2013) speculated that channel complexes embedded in cholesterol-rich nanoenvironments reorient in response to membrane stretch (strain) such that phospholipids dilute cholesterol regions, an event predicted to induce membrane thinning near the channel complex and subsequent MeT channel activation. Our results suggest that polyunsaturated acyl chains contained in PE and PS phospholipids are

required to modulate the mechano-electrical transduction process in TRN membranes. Further, they imply that touch-evoked membrane deformation and MeT channel activation would be compromised in mutants lacking C20 PUFAs in their acyl chains. Regulation of touch sensation by C20 PUFA-containing phospholipids may be conserved in mammals, including humans, because high dietary intake of PUFAs, including AA, is known to improve nerve conduction in older humans and lower the risk of peripheral neuropathy (Lauretani et al., 2007).

EXPERIMENTAL PROCEDURES

Strains

Worm culture and genetics were based on standard procedures (Brenner, 1974). Wild-type (N2) and mutant strains: BX24 *fat-1(wa9) IV*, BX30 *fat-3(wa22) IV*, BX17 *fat-4(wa14) IV*, BX107 *fat-5(tm420) V*, BX106 *fat-6(tm331) IV*, BX153 *fat-7(wa36) V*, BX52 *fat-1(wa9)*; *fat-4(wa14) IV*, and BX14 *elo-1(wa7) IV* were obtained from the Caenorhabditis Genetics Center, which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440). Null mutant TU253 *mec-4(u253) X* and transgenics TU3270 (*Punc-119::sid-1*; *Punc-119::yfp*; *Pmec-6::mec-6*) and TU3755 *uls58(Pmec-4::cfp, Pmec-4::mec-4::yfp)* were provided by Martin Chalfie (Columbia University). Strain TU3755 was crossed with BX52 and BX24 to obtain GN563 and GN564, respectively. Strain AQ2313 *ljls123(Pmec-4::ChR2 codon optimized; Punc-122::rfp)* was provided by William R. Schafer (Cambridge). AQ2313 animals were also crossed with *fat* mutants BX30 and BX52 to obtain GN439 and GN406, respectively. Null mutant *mboa-7(gk399)* was provided by Hiroyuki Arai (University of Tokyo) after out-crossing it five times.

A transgenic animal that expresses GFP under the control of *Pmec-17* (O'Hagan et al., 2005), TU2769 *uls31(Pmec-17::GFP) III* was crossed with *fat* mutant strains BX30, BX14, BX17, and BX52 to obtain GN212, GN214, GN215, and GN381, respectively.

Stable transgenic strains GN384 (40 ng/μl of *Pfat-3::gfp; Punc-122::rfp*; Watts et al., 2003), GN558 (10 ng/μl of *Pfat-4::gfp; Pmyo-2::mcherry; Punc-122::rfp*), and GN394 (40 ng/μl of *Pfat-1::gfp; Punc-12::rfp*) were obtained according to standard methods (Evans, 2006) by injecting the desired DNA construct and a cotransformation marker into N2 animals. At least three independent transgenic lines were analyzed for each construct; there was no detectable difference in the expression pattern of these lines.

Behavioral Assays

Gentle touch sensitivity was tested and scored as described (Hart, 2006). Briefly, ten-trial touch assays were performed by stroking a hair across the body and scored as percent response to anterior and posterior body touch; at least 25 animals were tested in each day's trial, and results were compared across three trials. All assays were performed blind to genotype and/or treatment.

Polyunsaturated Fatty Acid and Phospholipid Supplementation

AA (5,8,11,14-eicosatetraenoic acid) and EPA (5,8,11,14,17-eicosapentaenoic acid) were obtained from Nu-Chek Prep. ETYA (5, 8, 11, 14-eicosatetraenoic acid), AEA (N-[2-hydroxyethyl]-5Z,8Z,11Z,14Z-eicosatetraenamide), and 2-AG (5Z,8Z,11Z,14Z-eicosatetraen-2-glycerol ether) were acquired from Cayman Chemical. NGM was supplemented with PUFAs as previously described (Watts et al., 2003). Briefly, PUFAs were dissolved in ethanol and 0.1% Tergitol was added to NGM agar to reach, approximately, a 200 μM final concentration. Endocannabinoid AEA and 2-AG were dried with Ar gas, resuspended in DMSO, and added to NGM to reach, approximately, a 200 μM final concentration. 18:0-20:4 PC (1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine), 18:0-20:4 PE (1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine), and 18:0-20:4 PS (1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-L-serine) were obtained from Avanti Polar Lipids. Liposomes were prepared as previously described (Cortes and Perozo, 1997) and added to NGM to a final concentration of 100 μM. Plates were seeded with *E. coli* OP50 and kept at room temperature

for 2 days before the addition of embryos. Worm embryos were placed on fresh supplemented plates (≤ 1 week old).

Imaging

For promoter-fusion experiments, worms were paralyzed with polystyrene microspheres (Polysciences) and imaged with a 40 \times /1.25 oil immersion lens on a Leica SP5 confocal system. To evaluate TRN morphology and MeT channel distribution, worms were paralyzed on 1% agarose pads containing 150 mM of 2,3-butanedione monoxime and imaged with a 5 \times /0.15 air or 63 \times /1.4 oil immersion lens on Zeiss Axioplan 2 microscope.

Optogenetics

ATR was added to OP50 *E. coli* cultures and spread onto plates containing NGM (Nagel et al., 2005). Worm embryos were placed on fresh ATR plates (≤ 1 week old). Young adults were tested for withdrawal response in the presence and absence of ATR in the media. Worms were stimulated with blue light at 0.48 mW/mm², 470 nm for 1 s, every 30 s, at an intensity that produces saturating responses in wild-type worms. Neither ChR2 transgenics grown in the absence of ATR (Figure 4C) nor wild-type (N2) worms (data not shown) respond to this stimulus. Behavior was analyzed offline and defined as a worm reversal within 1 s of the light pulse and scored as percent in withdrawal response. All assays were performed blind to genotype and/or treatment.

RNAi Feeding

NGM plates were seeded with *E. coli* HT115, and double-stranded RNAi feeding was performed as previously described (Kamath et al., 2001). To knock down *mec-4* and *mboa-6* expression, wild-type (N2) and TU3270 adult worms were allowed to lay eggs for 2 hr on RNAi-containing plates, and the resulting progeny were tested for touch sensitivity as young adults. *mboa-6* RNAi was kindly provided by Hiroyuki Arai (University of Tokyo).

Primary Cell Culture and Atomic Force Microscopy

TRNs were isolated as described (Strange et al., 2007). Individual TRNs were visualized by GFP. AFM experiments were carried out essentially as previously described for embryonic zebrafish cells (Krieg et al., 2008) with a BioScope Catalyst BioAFM (Bruker NanoAXS) mounted on a Leica inverted microscope. Briefly, Olympus Biolevers (6 mN/m) were coated with 1 mg/ml peanut lectin in 4-morpholinethanesulfonic acid buffer (50 mM [pH 6]) overnight and calibrated according to thermal noise method before each experiment. Individual membrane nanotubes were pulled from isolated cells after 100–900 ms contact time and 400 pN contact force. Interaction frequency was adjusted so that 20% of cell-cantilever contacts yielded an event. This strategy ensures that only single-tether events were studied. Dynamic force-distance curves were analyzed using a step-fitting algorithm. The mean tether force versus extrusion velocity was plotted; we fit to the data the equation $f^3 - fF_0^2 = av$, where f is the tether force at a velocity v , F_0 is the static force, and a is the coefficient characterizing the dynamics of extrusion. This coefficient is defined as $a = (2\pi)^{3/2} \kappa^2 \eta \sqrt{\ln(R_c/R_t)}$, where κ is the plasma membrane bending rigidity, η is the membrane surface viscosity, and R_c and R_t are the radius of the cell and tether, respectively (Borghi and Brochard-Wyart, 2007; Brochard-Wyart et al., 2006; Diz-Muñoz et al., 2010).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.12.012>.

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