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MEMS-based Force-clamp Analysis of the Role of Body Stiffness in *C. elegans* Touch Sensation

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Abstract

Touch is enabled by mechanoreceptor neurons in the skin and plays an essential role in our everyday lives, but is among the least understood of our five basic senses. Force applied to the skin deforms these neurons and activates ion channels within them. Despite the importance of the mechanics of the skin in determining mechanoreceptor neuron deformation and ultimately touch sensation, the role of mechanics in touch sensitivity is poorly understood. Here, we use the model organism *Caenorhabditis elegans* to directly test the hypothesis that body mechanics modulate touch sensitivity. We demonstrate a microelectromechanical system (MEMS)-based force clamp that can apply calibrated forces to freely crawling *C. elegans* worms and measure touch-evoked avoidance responses. This approach reveals that wild-type animals sense forces $< 1 \mu\text{N}$ and indentation depths $< 1 \mu\text{m}$. We use both genetic manipulation of the skin and optogenetic modulation of body wall muscles to alter body mechanics. We find that small changes in body stiffness dramatically affect force sensitivity, while having only modest effects on indentation sensitivity. We investigate the theoretical body deformation predicted under applied force and conclude that local mechanical loads induce inward bending deformation of the skin to drive touch sensation in *C. elegans*.

Keywords

Mechanotransduction; touch; biomechanics; *C. elegans*; piezoresistor; MEMS cantilever; optogenetics; cuticle; touch receptor neurons; behavior

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INTRODUCTION

Touch sensation begins when a mechanical load is applied to our skin. The force generated is assumed to propagate through the skin, deforming the tissue and mechanoreceptor neurons that lie within it. Such deformation is believed to open ion channels in sensory neurons, triggering depolarization and, ultimately, activating a signaling network that informs our brain of the touch. The first step in touch, deformation of the tissues between the external environment and touch sensitive neurons, is critical in determining touch sensitivity. In support of this idea, human subjects with more conforming skin exhibit improved spatial resolution and roughness discrimination.^{1,2} Further, the mechanical properties of a patient's heel have been implicated along with decreasing touch sensitivity to result in accelerated plantar ulcer development among patients with diabetes mellitus.³ Despite these findings, our understanding of how changes in skin mechanics affect touch sensitivity is limited.

The nematode *Caenorhabditis elegans* is a powerful model for the study of touch. Gentle touch sensation in *C. elegans* is enabled by six touch receptor neurons (TRNs) that are embedded in the outer shell of the animal and run longitudinally down the body (Fig. 1). *C. elegans* responds to non-nociceptive (gentle) touch of its body with predictable responses: touching near the anterior TRNs of a forward moving animal will cause the animal to reverse direction. The TRNs are both necessary and sufficient to generate these responses: destroying the TRNs using genetic mutation or laser ablation abolishes touch-evoked avoidance responses,^{4,5} while optogenetic excitation of individual TRNs expressing channelrhodopsin elicits avoidance responses indistinguishable from those observed with touch stimuli.^{6,7} Physical deformation of the TRNs activates specialized mechanotransduction (MeT) channels, made up of the pore forming subunits MEC-4 and MEC-10 and several accessory proteins.^{8,9} These MEC-4-based MeT channels are distributed in discrete puncta along the length of each TRN, with an inter-punctum interval (IPI) of roughly 2 μm in wild type animals.¹⁰ Mechanical stimuli are believed to directly gate these channels and depolarize TRNs,⁸ an event that activates the neural circuit for avoidance responses.^{5,11}

Traditionally, *C. elegans* gentle touch is assayed by stroking a fine hair transversely across an animal's body.¹² This simple technique has dramatically advanced our understanding of touch by helping to identify a wide variety of proteins critical to touch through rapid behavioral screens of genetic mutants (*e.g.* Refs. 4, 13). However, it is not sufficient to determine the force delivered to *C. elegans*, since: 1) The force sensitivity of even very fine hairs is $> 1 \mu\text{N}$, 2) poor repeatability and significant uncertainty lead to variation between researchers and across experiments, and 3) it is impossible to maintain a constant force for defined time periods. More advanced techniques, such as the combination of *in vivo* electrophysiology and applied mechanical deformation with a glass micropipette on a piezoelectric actuator provide better stimulus control.^{8,14} For example, the force to generate a half maximal touch-evoked current in TRNs was $F_{1/2} \sim 0.75 \mu\text{N}$ when probes with a tip of diameter of 14-16 μm were indented into the worm's body.⁸ These techniques have advanced our understanding of the proteins involved in *C. elegans* gentle touch sensation and have given us a rough idea of the force sensitivity. However, these studies did not

directly measure or control the applied force and displacement and thus could not evaluate the contribution of body mechanics to *C. elegans* gentle touch sensitivity.

The outer shell of *C. elegans* is made up of the cuticle, the hypodermis, and the body wall muscles (Fig. 1). This structural element of the animal's body encases and protects vital organs and enables movement through the coordinated contraction and relaxation of body wall muscles. The TRNs are positioned just below the cuticle and embedded in the hypodermis. Internal hydrostatic pressure acts in conjunction with the outer shell to set the body's mechanics and stiffness. Previously, we used microelectromechanical system (MEMS) based piezoresistive microcantilevers to measure the stiffness of individual immobilized animals. We demonstrated that the mechanics of the outer shell are the dominant factor regulating overall body mechanics.¹⁶ Further, we showed that the body wall muscles (BWMs), an integral component of the outer shell, modulate body stiffness such that muscle hypercontraction renders animals both shorter and stiffer than they are at rest.¹⁷ Since the touch receptor neurons lie within the outer shell and this shell dominates overall body mechanics, increasing shell stiffness should decrease the deformation experienced by the TRN and correspondingly increase $F_{1/2}$ if deformation of the shell is critical for MEC-4 complex opening. Softening the shell should have the opposite effect.

In this study we directly test the hypothesis that modulating body mechanics affects touch sensitivity, with body stiffening increasing $F_{1/2}$ and vice-versa. To do this, we integrated a MEMS-based piezoresistive-cantilever force clamp system¹⁸ with a moving *x-y* stage, validated and calibrated the system to apply clamped forces and displacements to freely crawling animals, and used image processing to observe behavioral responses. For wild-type animals, we measured the probability of behavioral response over a range of forces at and below threshold and quantified $F_{1/2}$ and $\delta_{1/2}$, the force and indentation required to generate a half maximal response probability. We then leveraged two distinct and complementary approaches to modulate body mechanics with respect to wild-type: 1) disruption of the proteins DPY-5 and LON-2, required for normal cuticle structure and body morphology,^{19,20} and 2) optogenetically-induced body wall muscle hypercontraction. These manipulations allow the mechanics of the body to be altered without fully paralyzing animals such that behavioral assays are still possible. We find that softening the outer shell dramatically decreases $F_{1/2}$: *dpy-5* animals have the lowest stiffness and the smallest $F_{1/2}$, while BWM::ChR2 animals with light-induced muscle contraction have the highest stiffness and the largest $F_{1/2}$. In contrast, $\delta_{1/2}$ varies modestly with changes in body mechanics. These results support the hypothesis that changes in body mechanics induce changes in touch sensitivity and suggest that displacement of the outer shell drives gentle touch sensation in *C. elegans*.

RESULTS

Wild-type *C. elegans* respond to micro-Newton forces

To overcome the difficulties associated with applying minute forces and displacements to freely crawling *C. elegans*, we integrated a piezoresistive cantilever force clamp system capable of applying nN to μ N forces with a computer-controlled *x-y* stage and acquisition system (Materials & Methods). We used the *x-y* stage to position freely crawling young

adult or L4 larvae under the piezoresistive cantilever in real time and stimulated with forces between 100 nN and 10 μ N (Fig. 2). For each stimulus trial, we first held a designated force for 100 ms and then retracted the cantilever slightly so that the applied normal force was approximately zero (Materials & Methods). We chose the force application duration (100 ms) to be significantly longer than the rise time of the applied force (~ 10 ms) and the duration of measured mechanoreceptor currents generated in response to body indentation steps (~ 20 -50 ms),⁸ but as short as possible to minimize animal motion during stimulus application. The zero force position was held for 2 s, during which time behavioral responses were measured. Forces of 100 nN evoked reversal behaviors with a low probability, while forces above 1 μ N generated reversal behaviors with high probability (Fig. 2 E, F, G, and Supplementary Videos 1 and 2).

Disrupting cuticle proteins has little effect on TRN position or MEC-4 distribution

Since the mechanics of the outer shell of *C. elegans* dominate the overall body mechanics, we leveraged mutations in two genes encoding cuticle proteins: *dpy-5* and *lon-2*. Loss of *dpy-5*, which encodes a Group I cuticle procollagen, makes animals shorter and wider than wild type.¹⁹ By contrast, loss of *lon-2*, which encodes a heparan sulfate proteoglycan of the glypican subfamily,²⁰ makes animals longer and skinnier than wild type. On average, we found that *dpy-5* animals were much shorter and softer than wild type, while *lon-2* animals were slightly longer and softer (Fig. 3 A, B). The results for *dpy-5* animals agree with our prior investigation of immobilized animals, but our measurements here indicate a somewhat lower stiffness for freely crawling *lon-2* animals.¹⁶ We considered the possibility that the discrepancy was due to the bead at the tip of the cantilever slipping off of skinny *lon-2* animals and tested this idea by delivering ten 5 μ N stimuli to individual, freely crawling wild type, *dpy-5*, and *lon-2* animals and measuring the coefficient of variation (s.d. / mean) on an individual animal basis. If slippage were a significant factor, then we would expect that the variance would be increased for *lon-2* animals. We found no evidence of such a difference between wild type and *lon-2* animals, suggesting that a fundamental difference in stiffness may exist between freely crawling and glue-immobilized *lon-2* animals (Fig. 3C).

To investigate how these mutations affect TRN morphology, we measured their position as well as the distribution of the MEC-4-dependent MeT channels. We used transgenic animals expressing CFP and MEC-4::YFP under the control of a touch receptor neuron-specific promoter (*uls58*; J. Árnadóttir, *et al.*⁹) to visualize both the TRNs and the MEC-4 channel proteins in “wild type” animals. We crossed this transgene into the *dpy-5* and *lon-2* mutants in order to compare their TRNs and MEC-4 channel protein distribution to wild type animals. Despite significant differences in body morphology, TRN position (relative to other anatomical landmarks) was not significantly altered (Fig. 3D). For example, the anterior ALM touch receptor neurons innervated the body from roughly 5% to 43% of the body length from the nose in all three genotypes. Further, a MEC-4::YFP fusion expressed in the TRNs⁹ revealed that wild-type and *dpy-5* animals had indistinguishable inter-punctum interval, but that *lon-2* animals had a larger inter-punctum interval than wild type (Fig. 3E). On average, the inter-punctum interval was 14% larger in *lon-2* animals than in wild type, mirroring the average change in body and TRN length (13%). Collectively, these findings indicate that while loss of *dpy-5* or *lon-2* has a significant effect on *C. elegans* body size,

shape and mechanics, these mutations have little impact on TRN position and gross morphology.

Disrupting cuticle structure alters body stiffness and touch sensitivity

Since applied mechanical loads must be transferred from the body surface to the embedded touch receptor neurons, we reasoned that touch sensitivity would change in parallel with body stiffness. We tested this idea by measuring the response probability as a function of force in wild type, *dpy-5*, and *lon-2* animals. We used the piezoresistive force clamp system (See Materials & Methods) to apply calibrated forces between 100 nN and 10 μ N to freely crawling animals and observed behavioral responses. We applied a given force to a population of animals to quantify the probability of response. Repeating this procedure over a range of forces, we determined the probability of response as a function of force and fit an exponential function to this relationship (according to Weber's law, discussed by C. Mills, *et al.*²²) to estimate the force required to generate a half maximal response probability, $F_{1/2}$. Animals were studied on food-free agar substrates and each animal was subjected to one trial at a given force. We found that wild-type animals could detect sub- μ N forces, with an $F_{1/2}$ of approximately 0.5 μ N (Fig. 4A and Table 1). Consistent with the idea that cuticle stiffness affects force transmission, soft *dpy-5* mutants responded to smaller forces than wild type, with an $F_{1/2}$ of less than 0.1 μ N. Compared to wild type, *lon-2* mutants were slightly softer but had similar $F_{1/2}$. We also measured the indentation depth required to generate a half maximal response, $\delta_{1/2}$. By this criterion, *dpy-5* animals were more sensitive than wild type and *lon-2* animals (Fig. 4B and Table 1). Considered together with our findings that TRN position and MEC-4 inter-punctum interval are not significantly altered between wild type animals and *dpy-5* animals, these results support the hypothesis that body mechanics modulate touch sensitivity.

Optogenetic induction of muscle contraction increases body stiffness and reduces touch sensitivity

The body wall muscles (BWMs) are another critical component of the outer shell: they regulate body stiffness such that increasing muscle tone (hypercontraction) makes animals stiffer.¹⁷ Therefore, we sought to gain more insight into the relationship between body stiffness and touch sensitivity by acutely increasing muscle tone. To do this, we used the force-clamp system to compare the sensitivity of transgenic animals expressing the light-activated cation pump, channelrhodopsin2 (ChR2), specifically in body wall muscles to external mechanical loads before and during optical stimulation. Using a matched-pair approach, we subjected single BWM::ChR2 animals to ten trials at rest and ten trials while stimulated with blue light, alternating between the rest and stimulated states. The experiments were conducted with a thin layer of *E. coli* food on the plate to reduce touch desensitization over the duration of the experiment with repeated touch.²³ Exposing BWM::ChR2 animals to blue light contracted body wall muscles, decreased body length and increased body stiffness (Fig. 5A). The observed relationship between the change in body length and change in body stiffness between the rest and hypercontracted states agreed well with prior measurements of immobilized animals (change in stiffness / change in length = $k/l = -2.2\%$ previously in glued animals, $k/l = -3.0\%$ here).¹⁷ Animals

stimulated with blue light retained their mobility despite undergoing BWM hypercontraction.

Blue light-induced muscle hypercontraction and body stiffening sometimes, but not always, decreased sensitivity to the next stimulus (Fig. 5B). Across a population of animals stimulated with force pulses between 0.5 and 5 μN , we observed a half-maximal response force ($F_{1/2}$) of less than 1 μN in the resting state (Fig 5C). Muscle hypercontraction significantly increased $F_{1/2}$. This increase in $F_{1/2}$ was reversible and repeatable: shifting from light-off to light-on and *vice versa*, revealed that as stiffness increased with muscle hypercontraction, the probability of response decreased for nearly all force levels and trials (Fig. S1). We observed a similar, but less dramatic increase in $\delta_{1/2}$ with increases in body stiffness (Fig. 5D). Although muscle hypercontraction decreased the probability of response at a given force or displacement magnitude, the quality of the responses, assessed by response latency, were not significantly different between the rest and muscle hypercontracted states (Fig. 5E). These findings support the hypothesis that body mechanics modulate force sensitivity with muscle hypercontraction and associated body stiffening leading to an increase in $F_{1/2}$ and vice-versa.

DISCUSSION

In this work, we used two distinct and complementary methods, genetic mutation of cuticle proteins and optogenetic modulation of body wall muscle tone, to demonstrate that body mechanics affect touch sensitivity in the nematode *C. elegans*. However, mechanics play an important role not only in *C. elegans*, but also in humans. For example, investigations in human touch have emphasized the likely importance of skin mechanics in determining sensitivity.¹⁻³

Although we have focused on mechanics, the potential importance of TRN morphology should not be overlooked. Our findings indicate that TRN position does not vary significantly between wild-type, *lon-2(e678)*, and *dpy-5(e61)* animals, despite significant differences in body length. This finding suggests that the gross morphology and position of the TRNs are independent of cuticle structure and body size. Our studies of MEC-4 interpunctum interval (IPI) further reveal that the MEC-4 IPI is similar in wild type and *dpy-5* animals, but roughly 14% larger in *lon-2* animals. These findings suggest that there are both cell-autonomous and cell-nonautonomous factors that regulate MEC-4 complex localization. With an increase in IPI, we expect force and indentation depth sensitivity to decline since the number of addressable channels under a given deformation decreases. This effect could underlie the similarity between wild type and *lon-2* sensitivity. That is, if only mechanics were important, *lon-2* animals should be more force sensitive (smaller $F_{1/2}$) than wild type animals since they are softer. However, we observed that *lon-2* worms have both similar $F_{1/2}$ and $\delta_{1/2}$ to wild type. Thus, the increased IPI in *lon-2* animals may compete with the predicted decrease in $F_{1/2}$ with a softer body shell.

In general, the studies presented here indicate that increasing body stiffness increases both $F_{1/2}$ and $\delta_{1/2}$, and vice versa. These findings support our hypothesis that body mechanics affect touch sensitivity in *C. elegans*. To gain more insight into whether animals detect the

forces delivered by mechanical loads or respond to the indentations they produce, we plotted the measured $F_{1/2}$ and $\delta_{1/2}$ versus body stiffness across all strains and conditions. If force were the proximal stimulus (i.e., the parameter measured directly by the TRNs), then we expect that very soft and very stiff worms would have the same half-activation force, $F_{1/2}$, regardless of the fact that the soft worm would experience larger indentations than the stiff worm. Contrary to this expectation, we found that body stiffness was more strongly correlated with $F_{1/2}$ than with $\delta_{1/2}$ (Fig. 6). Collectively, these results suggest that animals respond more directly to the indentation generated by mechanical loads rather than the force carried by such loads.

Additional insight into the relationship between indentation depth and behavioral response probability can be gained by considering the primary deformation modes of the worm's body. Given our prior findings that the outer shell (consisting of the cuticle, the hypodermis and the body wall muscles) of the animal dominates the overall body stiffness,¹⁶ there are two deformation modes that contribute to measured body indentation: 1) local deformation of the outer shell at the worm-bead interface, and 2) global deformation of the outer shell (Fig. 2C). The local deformation can be approximated using Hertzian contact mechanics for small indentation depths ($\delta_{local} < 1 \mu\text{m}$ for $R_{bead} = 5 \mu\text{m}$). Modeling the contact as a sphere indenting an infinite half-plane, we have

$$F_{local} = \frac{4}{3} \Xi^* R_{bead}^{1/2} \delta_{local}^{3/2} \quad (1)$$

where $\Xi^* = \Xi / (1 - \nu^2)$, Ξ is the effective Young's modulus and ν is Poisson's ratio. In this case, the local tangent stiffness is

$$k_{local, tng} = \frac{\partial F_{local}}{\partial \delta_{local}} = 2 \Xi^* R_{bead}^{1/2} \delta_{local}^{1/2} \quad (2)$$

With increasing indentation depth, $k_{local, tng}$ scales with $\delta_{local}^{1/2}$, increasing rapidly over the range of indentation depths over which Hertzian theory applies. The other deformation mode is global collapse of the cylindrical outer shell of the animal. The relationship between force and indentation with this mode can be approximated as^{16,24}

$$F_{global} = \frac{1.37 \Xi t^{5/2} \delta_{global}}{R_{worm}^{3/2}}, \quad (3)$$

where t is the effective thickness of the outer shell and R_{worm} is the radius of the animal, as long as $t \ll R_{worm}$. In this case, stiffness is predicted to be constant over the entire range of forces, with

$$k_{global} = \frac{F_{global}}{\delta_{global}} = \frac{1.37 \Xi t^{5/2}}{R_{worm}^{3/2}} \quad (4)$$

Since the local and global deformation modes operate in series, if we apply a force F to *C. elegans* and measure the total deformation (δ), we have two additional relationships:

$$F_{local}=F_{global}=F, \text{ and } (5)$$

$$\delta=\delta_{local}+\delta_{global}. (6)$$

We can leverage these relationships to solve for the global deformation by starting with Equation 5, plugging in the local and global forces, then using Equation 6 to substitute $\delta_{global}=\delta-\delta_{local}$:

$$\frac{\delta_{local}^{3/2}}{\delta-\delta_{local}}=\frac{1.03(1-\nu^2)t^{5/2}}{R_{bead}^{1/2}R_{worm}^{3/2}}. (7)$$

Previously, we estimated $t \sim 1.4 \mu\text{m}$,¹⁷ and measured $R_{worm} = 18 \mu\text{m}$ ¹⁶ for L4 wild type animals. With $R_{bead} = 5 \mu\text{m}$, and assuming $\nu = 0.5$ (incompressible material), Equation 7 then predicts that local deformation will contribute less than 20% of the measured indentation for indentations (δ) between 10 nm and 10 μm . This result suggests that global deformation, and not local deformation, dominates under these conditions. For example, for a 1 μm indentation, Equations 6 and 7 yield $\delta_{local} = 0.05 \mu\text{m}$ and $\delta_{global} = 0.95 \mu\text{m}$. Even when the local indentation is more accurately modeled by correcting for the worm's cylindrical shape, global deformation will still dominate.²⁵

Importantly, the foregoing analysis does not depend on the effective Young's modulus (Ξ) of the animal. This will be the case as long as our assumptions that $\delta_{local} < 1 \mu\text{m}$ and that $t \ll R_{worm}$ are met. Thus, our conclusion that global deformation dominates would be appropriate regardless of whether our previous estimate (10 MPa, in *BWM::Chr2* animals at rest; B. C. Petzold, *et al.*¹⁷), or another estimate of the effective Young's modulus of the outer shell of *C. elegans* is more accurate.^{26,27} This theoretical analysis suggests that the observed increase in sensitivity with decreasing body stiffness is not brought on by increasing contact area of the bead with increasing force, but rather global deformation of the entire outer shell of the worm.

CONCLUSION

We developed a force clamp behavior system capable of applying calibrated forces to freely crawling *C. elegans* to measure the force and displacement sensitivity of touch-evoked behavioral responses. We demonstrated that wild type animals respond to forces $< 1 \mu\text{N}$ and indentation depths $< 1 \mu\text{m}$ within 2 seconds by reversing movement direction. We used the system and two methods, optogenetics and genetic mutation of cuticle proteins, to directly test the hypothesis that body mechanics affects touch sensitivity. We found that *BWM* hypercontraction and mutation of cuticle proteins result in large changes in body mechanics, as previously reported,^{16,17} and that the corresponding changes in body stiffness resulted in dramatic changes in force sensitivity. In general, stiffening the body increased $F_{1/2}$, while softening the body decreased $F_{1/2}$. Changes in displacement sensitivity followed the same relationship, but were less dramatic, suggesting that indentation depth is a more proximal touch stimulus than force. Estimated local stiffness is theoretically much greater than global deformation stiffness, suggesting that, when indenting *C. elegans* with a 10 μm bead, the

force can be approximated as a point load. Further, we implicate a role for MEC-4 inter-punctum interval, which may set both force and indentation sensitivity in conjunction with body mechanics.

In unraveling the mechanisms underlying touch and its disruption in humans and *C. elegans* alike, there is a critical need to understand the relationship between tissue mechanics and touch sensitivity. This study highlights how dramatically force sensitivity can vary with modest changes in body stiffness in *C. elegans*. Our ability to model and understand force transfer and shell deformation could be enhanced by future studies investigating the mechanical properties of individual components of the outer shell of *C. elegans*. Further, the theoretical model of *C. elegans* body wall deformation under applied force could be tested by performing force clamp behavior experiments with beads of different radii: body stiffness is not predicted to change significantly with variation in bead diameter when global deformation dominates. Improvements to the force clamp system including automated sample tracking and improved data analysis algorithms could minimize the need to reject data (see Materials & Methods), increase throughput, and enable application of the force clamp system to a wider range of samples and experiments. An improved understanding of force transfer and shell deformation in *C. elegans* touch sensation can be combined with TRN morphology studies, and electrophysiological studies of the touch-sensitive ion channels themselves to shed new light on the mechanisms that enable touch sensation.

EXPERIMENTAL

Strains

The following *C. elegans* strains were used: wild-type (N2), CB61 *dpy-5(e61)I*, CB678 *lon-2(e678)X*, GN362 *oxIs353[Pmyo-3::Chr2::mCherry + lin-15(+)] + Litmus[V]*; *lite-1(ce314)X*, GN402 *uls58[Pmec-4::CFP, Pmec-4::MEC-4::YFP]*, GN416 *dpy-5(e61)I; uls58[Pmec-4::CFP, Pmec-4::MEC-4::YFP]*, and GN417 *lon-2(e672)X; uls58[Pmec-4::CFP, Pmec-4::MEC-4::YFP]*. N2, CB61, and CB678 strains were obtained from the Caenorhabditis Genetics Center; *uls58* was a gift from M. Chalfie. For clarity, we refer to strain GN362 as BWM::Chr2 and *uls58* as MEC-4::YFP. Animals were maintained using standard procedures,²⁸ synchronized according to Hope, *et al.* (Ref. 29), and grown at 20°C until reaching the L4/young adult stage. Only animals in the L4/young adult stage were used in order to ensure that the anatomy, particularly of the outer shell, was held constant. This is critical since the cuticle, among other things, changes dramatically between growth stages.³⁰ To activate Chr2 in BWM::Chr2 animals, animals were grown in the dark and in the presence of 500 μ M all-trans retinal (Sigma Aldrich, St. Louis, MO, USA) as in Nagel, *et al.*³¹

Sample and Substrate Preparation

Behavior studies—Measurements were performed on 1.8% (wt/vol) NGM agar pads with 3 mg/mL NaCl, 10 mg/L cholesterol, 1 mM CaCl₂, 1 mM MgSO₄, and 25 mM KH₂PO₄ (pH 6.0) with an osmolarity of approximately 182 mOsm. Pads were roughly 1 cm² in area and 1 cm thick. Agar pads were placed in a shallow dish lined with damp paper towels to minimize dehydration. The agar was food-free for studies of N2, *dpy-5* and *lon-2*,

but carried a thin layer of *E. coli* OP50-1 for studies of BWM::ChR2. We created thin lawns by dispensing ~100 μ L of *E. coli* broth onto each 35 mm NGM agar plate and allowing it to dry by a flame at room temperature. *TRN morphology*: Measurements were performed on thin 6% (wt/vol) NGM agarose pads. Animals were immobilized by depositing 2 μ L of 0.1 μ m polystyrene beads in solution (#00876-15, 2.5% w/v suspension, Polysciences, Inc., Warrington, PA, USA) onto the surface, picking a worm into the solution, and covering the sample with a #1.5 coverslip.

Experimental Techniques

Piezoresistive cantilever force-clamp behavior studies—The MEMS-based piezoresistive cantilever force-clamp system has been described in detail previously.¹⁸ Here we expand system functionality by integrating a motorized *x-y* stage to position samples under the cantilever and by using image processing adapted from Leifer, *et al.* (Ref. 6), to score *C. elegans* behavior. Briefly, single-crystal silicon piezoresistive microcantilevers (typical dimensions: 1000 μ m long, 30 μ m wide, 7 μ m thick, stiffness, $k_c \sim 0.4$ N/m) with a glass bead (diameter = 10 μ m) at their tip are mounted on a piezoelectric actuator. We used 10 μ m-diameter glass beads for consistency with prior work^{8,17,18} and because 10 μ m is smaller than the body diameter of *C. elegans* (~30 μ m) but large compared to the average spacing of MEC-4 ion channel complexes along each TRN (~2 μ m). Cantilevers were calibrated before each set of experiments to accurately measure displacement sensitivity. A real-time controller operating at 100 kHz measures the deflection of the cantilever, z_c , and the actuator position, z_a , and adjusts the actuator position to clamp the force, $F = k_c z_c$, applied to the sample (Fig. 2). The sample is positioned under the cantilever with the *x-y* stage; stage motion is enabled by two linear actuators with 25 mm maximum travel (T-NA08A25s, Zaber Technologies, Vancouver, Canada) and controlled with a computer interface. The operator targets the stimuli location and triggers the real-time controller to apply a pre-selected force via a mouse-click on the live image.

For behavior studies, we placed *C. elegans* on an agar substrate on the *x-y* stage. An individual animal was positioned under the tip of the cantilever in real time. The animal was then stimulated with a three-step force profile: loading, “zero force,” and unloading (Fig. 2E). The force range for this study was smaller than the surface interaction force.³² In particular, stiction forces between *C. elegans* and the glass bead at the cantilever tip elicit behavioral responses when the cantilever is unloaded. To avoid this possibility, we added a 2 s zero force step between the loading and unloading steps. In the “zero force” step, the applied force was on the order of the resolution of the piezoresistive microcantilever (sub-nN). We confirmed that *C. elegans* can move forward normally (Supplementary videos 1 and 2) during the “zero force” step. Thus, we were able to monitor behavioral response to a set force during this 2 s step, while minimizing potential confounding due to the relatively large surface interactions and capillary forces near the hydrated surface. A typical force clamp behavior experiment involves at least 10 trials of force pulses between 100 nN and 1 μ N and lasts about 30 minutes. The primary failure mode (rejected data) is mistargeting: *i.e.* the user misses the animal’s body with the bead. Experienced users miss their target less than 20% of the time, while new users may miss in more than 50% of trials. This failure rate

could be improved in the future by integrating the force clamp system with one of the automated sample tracking technologies recently demonstrated for *C. elegans* assays.^{6,7}

To analyze the behavioral response to force, we screened the responses manually but also conducted image post-processing. We calculated each animal's body curvature waveform by adapting previously developed image analysis software.⁶ In particular, we removed the cantilever's shadow by obtaining the skeleton curves from digitized binary images and considering only the skeleton curve that directly connected the head and tail of the animal (Fig. S2). Removing the cantilever's shadow is one of the major challenges that needs to be overcome to integrate the force clamp system with automated sample tracking.^{6,7} We used kymographs to display body curvature as a function of distance along the centerline and time.²¹ Figure 2 shows kymographs of the time-varying body curvatures and associated trajectories for wild-type animals. Local body curvature, κ , was calculated at 20 points evenly spaced along the parameterized body centerline, $c(s) = (x(s), y(s))$, as

$$\kappa(c(s)) = \frac{|x'y'' - y'x''|}{(x'^2 + y'^2)^{3/2}}. \quad (6)$$

The recorded actuator and cantilever signals allowed a wide array of parameters, including indentation depth ($z_s = z_a - z_c - z_{initial}$), body stiffness, and others to be determined in addition to the behavioral parameters gathered with image processing. Body stiffness, k_s , was calculated as

$$k_s = \frac{F}{z_s} = \frac{k_c z_c}{z_a - z_c - z_{initial}}. \quad (7)$$

Positive responses to an applied force were scored if the animal backed up before the cantilever was pulled off (after 2 s); pauses were not counted as responses. Trials where the cantilever slid off of the worm's body during the 100 ms force application were not counted. In this study, we focused on the response to stimuli delivered to the anterior body. To achieve this goal, we included trials in which the stimulus was applied between the terminal bulb of the pharynx and the vulva (between roughly 25 and 50% of body length from the tip of the nose) and omitted all other trials.

ChR2 Excitation—A Xenon lightsource (DG-4+, Sutter Instrument Co., Novato, CA, USA) was integrated with the force-clamp system to enable computer-controlled shuttering of the light and synchronized recording of whether or not the light was on in all videos. We altered our previous force-clamp behavior rig, adding a fluorescence turret with a standard GFP (GFP2 Filter Set, Leica Microsystems Inc., Buffalo Grove, IL, USA) filter set and a Leica PLANAPO 1.0x objective with a 0.63× C-mount to maximize fluorescence intensity at the sample without altering the field of view. This combination of optical components enabled blue light intensities between 0.66 and 1.0 mW/mm², as measured by a FieldMate laser power meter with Coherent PowerMax PM10 sensor (Coherent Inc., Santa Clara, CA, USA).

TRN position measurement—Individual GN402, GN416 and GN417 worms were imaged in both brightfield and CFP fluorescence on a Leica DMIRB inverted microscope with a Hamamatsu Orca-R2 camera (Hamamatsu, Hamamatsu City, Japan) with 10x and 20x air objectives (Leica). An ECFP filter (Leica) was used to measure CFP fluorescence. Images from several focal planes were taken to capture all of the TRNs. Some worms were too large to be completely captured in a single field of view at the magnifications used to image the TRNs. In these cases, the anterior portion of the worm was first imaged both in brightfield and then in fluorescence while keeping the lateral position of the stage constant. The posterior portion of the worm was then imaged in the same fashion. Adobe Photoshop CS5 was used to create a brightfield montage. Then each fluorescence image was overlaid on top of the pixels from the corresponding brightfield image (Fig. S3). A region of interest along the spline of the complete brightfield image of the worm was defined manually in ImageJ. This same region of interest was applied to each of the fluorescence images. Then each spline was straightened in ImageJ. Since the same translation was used on each image, the pixels in each straightened image all mapped to the same points on the brightfield image (Fig. S4). From these straightened images, the position of the center of each TRN cell body, the end of each TRN and the point at which AVM and PVM made a junction at the sidewall of the worm's body were recorded. The position of the head and tail were also recorded. We mapped the position of each TRN feature to the percentage down the length of the body from the head.

MEC-4 inter-punctum interval measurement—The MEC-4::YFP puncta of individual GN402, GN416 and GN417 animals were imaged using a Nikon TiE inverted microscope (Nikon Instruments, Inc., Melville, NY, USA) equipped with a 2× multiplier and a Nikon 40× oil-immersion objective, an 89 North Heliophor LED light source using the 500 nm module (89 North, Burlington, VT, USA), a 560/25 nm BrightLine® single-band bandpass filter (FF01-560/25-25) and 520 nm dichroic (Semrock Inc., Rochester, NY, USA) filter set, and an Andor Neo sCMOS camera (Andor Technology, South Windsor, CT, USA). With a 1× C-mount, this combination of optical components had spatial resolution limited by the diffraction limit of ~ 200 nm. Images of individual neurons were imported into ImageJ, straightened, and background subtracted. The resulting straightened MEC-4 puncta intensity maps were converted into intensity vs. position by recording the intensity along the midline of the TRN. These data were then imported into Matlab®, where a peak finding algorithm with a smoothing width of 0.5 μm was used to measure each inter-punctum interval down the length of the TRN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *C. elegans* gentle touch is enabled by six touch receptor neurons (TRNs) embedded in the outer shell of the body

(Top) Each TRN innervates roughly 50% of the body length. Since wild type *C. elegans* crawls on its side, ALMR/L and PLMR/L are near the apex of the body at all times.

(Bottom) A cross-section through the body plan reveals the key elements of the outer shell: the cuticle, the hypodermis, and the body wall muscles (not to scale). The internal anatomy and organs is not shown. The TRNs are embedded in the hypodermis just below the cuticle. Approximate TRN positions are from Altun, *et al.*¹⁵ Body plan coordinates: *a* – anterior, *p* – posterior, *v* – ventral, *d* – dorsal, *r* – right, *l* – left.

Figure 2. Integrated piezoresistive cantilever force-clamp and behavioral analysis system

(A) Schematic diagram of the system which is composed of a piezoresistive microcantilever mounted on a printed circuit board and moved in the z -direction by a piezoelectric actuator, a video camera for real-time visualization and *posthoc* behavioral video analysis, and a high-intensity light source for optogenetics. Actuator position is under feedback control to enable delivery of defined forces (see Methods). Elements marked with asterisks (*) are connected to computers for control and data acquisition. (B) Still image from a behavioral experiment. The bead at the tip of the cantilever is positioned over the anterior portion of a forward moving animal. (C) Applied force is determined from the measured cantilever deflection (z_c) and calibrated cantilever stiffness (k_c). Deflection of the animal's body surface consists of both local (at the bead-cuticle interface) and global deformation. (D) The force clamp system can deliver forces from nNs to tens of μ Ns. (E) For behavior experiments, a designated force is held for 100 ms, then the cantilever is retracted to hover above the animal and deliver no force for a 2 s observation period. Micrographs are a series of still images showing a wild-type worm responding to a force of 1 μ N (top), but not responding to a force of 0.1 μ N (bottom). (F) Body curvature matrices record curvature as a function position along the body's length (y-axis: top is $l = 0\%$ of body length from head, bottom is $l = 100\%$) as a function of time.²¹ Red color indicates dorsal bending; blue indicates ventral bending. More intense colors indicate larger curvature. For a 1- μ N stimulus, the matrix on the right illustrates a change in movement direction of the animal. (G) Head position plotted versus time also depicts a change in movement direction with 1 μ N of force.

Figure 3. Genetic manipulation of cuticle proteins alters body mechanics, but not TRN morphology

Deleting *dpy-5* or *lon-2* alters (A) body morphology, and (B) body length and stiffness. Data are mean $\pm$ s.e.m. of at least 146 animals. * indicates $p < 0.01$ (student's t-test with a Bonferroni correction for 6 comparisons). (C) The coefficient of variation for a 10 touch stiffness measurement is similar for wild type and *lon-2* animals, but smaller for *dpy-5* animals. Boxes represent 25th-75th percentiles. Thin line is median, thick line is mean ($n = 5$ animals). * indicates $p < 0.05$ (student's t-test with a Bonferroni correction for 3 comparisons). (D) The position of anterior TRNs is similar in wild type, *dpy-5* and *lon-2* animals (not significant, student's t-test with a Bonferroni correction for 6 comparisons). The micrograph shows CFP-labeled anterior touch receptor neurons (ALM, AVM) in a *uls558* animal. Points are mean $\pm$ s.e.m. of at least 4 animals. (E) MEC-4 inter-punctum interval is similar in wild type and *dpy-5* mutants, but larger in *lon-2* mutants. Micrograph shows MEC-4::YFP puncta in anterior TRNs in a *uls558* animal. Points are mean $\pm$ s.e.m. of at least 208 IPIs from the TRNs of at least 3 animals. * indicates $p < 0.05$ (student's t-test with a Bonferroni correction for 3 comparisons). Some error bars are smaller than data points.

Figure 4. Genetic manipulation of cuticle proteins alters touch sensitivity

(A) $F_{1/2}$ differs in *dpy-5* animals with respect to wild type, but is similar among *lon-2* and wild type animals. (B) $\delta_{1/2}$ does not vary in *dpy-5* or *lon-2* animals with respect to wild type. Data are mean $\pm$ s.e.m. of the probability of response of a population of at least 7 animals at each force or indentation level. Wild-type data are shown with dashed lines for clear differentiation. Lines are exponential fits with a horizontal offset of the form $p(x) = be^{(x-c)/a}$, weighted by the inverse variance of each force or indentation sample. Finely dashed vertical lines highlight $F_{1/2}$ and $\delta_{1/2}$ for each strain; values and their errors are given in Table 1. * indicates $p < 0.05$ for differences between $F_{1/2}$ or $\delta_{1/2}$ (student's t-test with Bonferroni correction for 3 comparisons).

Figure 5. Optogenetic induction of body stiffening decreases touch sensitivity

(A) Blue light triggers muscle hypercontraction BWM::ChR2, shortening and stiffening the body. Data are mean $\pm$ s.e.m. ($n = 20$). * indicates $p < 0.05$ (student's t-test with Bonferroni correction for 2 comparisons). (B) Muscle hypercontraction sometimes (top row), but not always (bottom row), reduces sensitivity to defined forces. Body shortening results in a reduction in the height of the curvature matrix in the second column. (C) BWM::ChR2 animals experience an increase in $F_{1/2}$ and (D) $\delta_{1/2}$ when exposed to blue light. Data are mean $\pm$ s.e.m. of at least 3 individual animal response probabilities at each force level. Lines are exponential fits with a horizontal offset of the form $p(x) = be^{(x-c)/a}$, weighted by the inverse variance of each force or indentation sample. Finely dashed vertical lines highlight $F_{1/2}$ and $\delta_{1/2}$ for each condition; values and their errors are given in Table 1. * indicates $p < 0.05$ for differences between $F_{1/2}$ or $\delta_{1/2}$ (student's t-test). (E) The responses of BWM::ChR2 animals have similar latency in both the rest and muscle-hypercontracted states. Boxes represent 25th-75th percentiles. Thin line is median, thick line is mean. + 's indicate outliers.

Figure 6. Body stiffening significantly increases the force required for half-maximal response probability to touch, but only moderately increases the required indentation
 $F_{1/2}$ increases with body stiffness under all conditions examined in this study (top: line is a linear fit to $\log(F_{1/2})$ vs stiffness, k . $R^2 = 0.84$. Statistical significance, intercept: $p = 0.023$, slope: $p = 0.028$, two-sided linear regression t-test). By comparison, half-maximal indentation, $\delta_{1/2}$, is less dependent on stiffness, k (bottom: $R^2 = 0.63$. Statistical significance, intercept: $p = 0.09$, slope: $p = 0.11$, two-sided linear regression t-test). Data are mean $\pm$ s.e.m. (see Table 1). Some error bars are smaller than data points.

Table 1

Body length, body stiffness, and force and indentation sensitivity of wild type and mutant *C. elegans*.

Strain	Body Length (μm) [*]	Body Stiffness, k_s (N/m) [*]	$F_{1/2}$ (μN) [†] $\delta_{1/2}$	(μm) [†]
wild type (N2)	718 $\pm$ 3	0.75 $\pm$ 0.01	0.49 $\pm$ 0.10	0.44 $\pm$ 0.13
<i>dpy-5(e61)</i>	470 $\pm$ 4	0.39 $\pm$ 0.02	0.064 $\pm$ 0.016	0.19 $\pm$ 0.029
<i>lon-2(e672)</i>	819 $\pm$ 2	0.67 $\pm$ 0.01	1.09 $\pm$ 0.30	0.82 $\pm$ 0.067
BWM::ChR2, rest	684 $\pm$ 11	0.67 $\pm$ 0.04	0.73 $\pm$ 0.28	1.30 $\pm$ 0.22
BWM::ChR2, hypercontracted	639 $\pm$ 9	0.80 $\pm$ 0.06	2.24 $\pm$ 0.15	2.63 $\pm$ 0.21

^{*} Values are mean $\pm$ s.e.m. Sample sizes were at least 108 animals for wild type, *dpy-5*, and *lon-2* animals, and 20 animals for BWM::ChR2.

[†] Values are mean $\pm$ s.e.m. Errors are derived from the errors in the exponential fits to the p vs F or δ data. Population responses to at least 5 forces or indentations were used.