Modulation of touch sensitivity in *Caenorhabditis elegans*

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Abstract

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Sensory perception adapts to diverse environment. Although studies in the last few decades have started to address the question of how sensory systems transduce signals, how these systems cross-modulated is largely unknown. In this thesis, I study mechanosensation in the *C. elegans* touch receptor neurons (TRNs) to understand how sensory systems are modulated and adapt to the environment. I find that the touch sensitivity in the TRNs is modulated by both mechanical and non-mechanical factors. The mechanical factors are transduced directly by a secondary mechanosensory system in the TRNs, and the non-mechanical factors are detected by other neurons and relayed to the TRNs by neuropeptides. Both pathways converge through a common mechanism to regulate the surface expression of the MEC-4 mechanotransduction channels, which are needed for sensing touch. I then explore the consequences of modulation, and show that modulation by mechanical and non-mechanical factors adjusts the balance between the sensitivity to strong mechanical stimuli that predict dangers and sensitivity to weak stimuli that are usually not associated with danger. Such a balance maintains sensitivity to biologically-relevant mechanical stimuli while reducing unnecessary responses to weak stimuli, thus increasing the ability to survive under different conditions.

I used neuronal-enhanced RNAi and mosaic analysis to discover two convergent signaling pathways, the integrin/focal adhesion signaling and insulin signaling, that modulate anterior touch sensitivity. Additional genes and pathways are also needed for

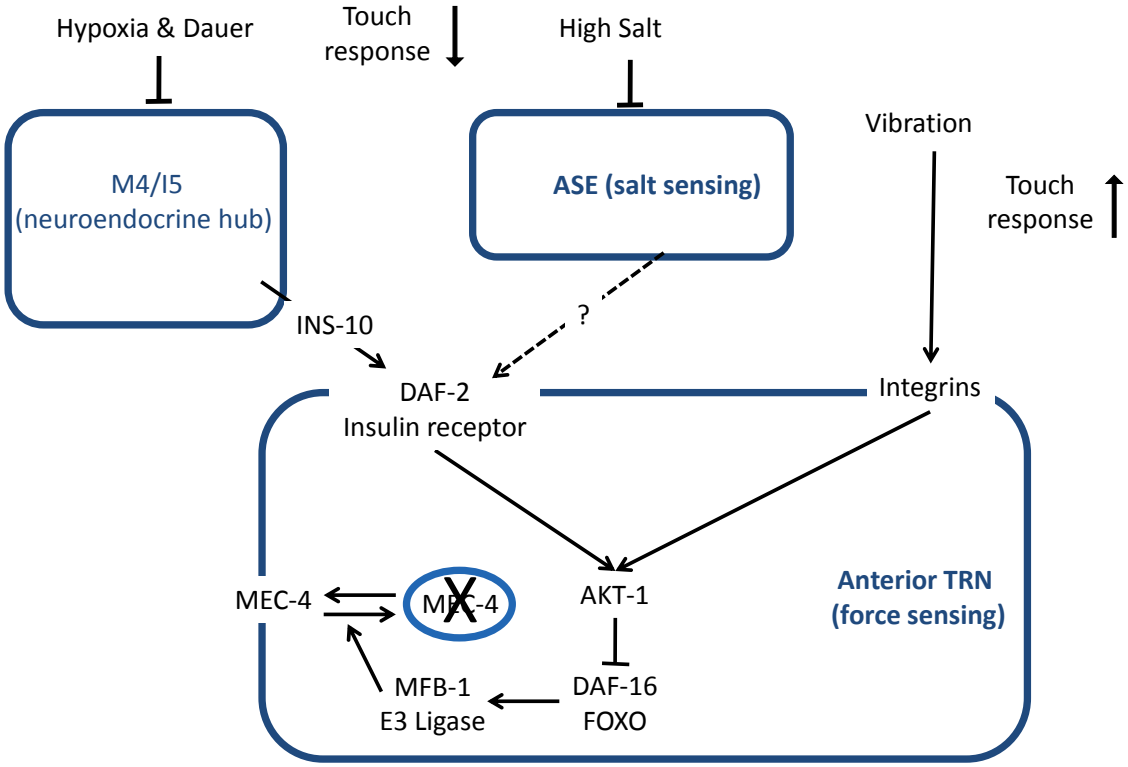
optimal touch sensitivity in the TRNs, including the RAS/MAPK pathway, Rho-GTPases, cytoskeleton genes, and 43 other genes that cause lethality when mutated.

The integrins/focal adhesion proteins act cell-autonomously in the TRNs to detect the mechanical environment. The focal adhesion proteins modulate force sensitivity and subsequent calcium signaling, and they are needed for long-term sensitization of touch sensitivity in response to sustained background vibration. Such sensitization maintains normal touch sensitivity under background vibration by partially counteracting the effect of habituation. This sensitization does not require the MEC-4/MEC-10 transduction channel, suggesting that the integrins may act as secondary force sensors.

Insulin signaling, however, responds to non-mechanical signals that reduce touch sensitivity by decreasing the expression of insulin-like neuromodulators, including INS-10 and INS-22. The reduced touch sensitivity facilitates the completion of other tasks such as chemotaxis under background mechanical stimuli, thus increasing the chance of survival by escaping stressful conditions.

Both insulin signaling and integrin signaling converge on AKT-1 and DAF-16, which modulate touch sensitivity by regulating the transcription of *mfb-1*, an E3 ubiquitin ligase expressed in the TRNs. MFB-1 regulates the amount of MEC-4 channel on the plasma membrane, thus modulating touch sensitivity. Together, these results describe an integrated pathway that transduces both mechanical and non-mechanical signals to modulate touch sensitivity through a common mechanism. These modulation mechanisms maintain optimal sensitivity to mechanical stimuli while avoiding unnecessary responses.

Graphical abstract



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Chapter I. Introduction

(This chapter is arranged in two sections. The first section (I-A) provides background information about sensory modulation. The second section (I-B) introduces mechanosensation and its modulation.)

Animals adapt to survive in a wide range of environments. This ability largely depends on an animal's amazing ability to perceive its diverse surroundings through sensory perception. The signals that an animal senses usually include mechanical stimuli (hearing, touch sensing, somatosensation and gravity sensing), chemical stimuli (olfaction, gustation and pheromone detection), temperatures, and light (vision) (Kandel et al., 2000). Some species can detect additional information such as magnetic fields (Wiltschko and Wiltschko, 1996) or electric fields (Lissmann, 1958). Combining information from these senses creates a representation of the outside world that facilitates the survival of the animal.

However, one problem associated with sensory systems is that they need to function under diverse conditions, and only certain stimuli represent biologically relevant information. For example, mammalian eyes detect signals from almost complete darkness to bright sunlight, differing by ten orders of magnitude. However, the information relevant to an animal is usually not encoded in the absolute brightness of the environment, but in differences in the brightness, i.e., contrast and patterns. Similarly, the olfactory system can detect changes in one compound while being habituated to another constantly present compound, therefore extracting only information that may be relevant to the animal (Colbert and Bargmann, 1995). The ability to extract useful information from background stimuli requires modulation of sensory perception in different environments.

Sensory perception can be modulated directly by current and/or previous activities of the sensory system, which usually facilitate the accurate sensing of useful signals in the presence of ambient signal or noise by adjusting the sensitivity and/or gain of the

system. In addition, sensory perception can be modulated by other sensory systems or even motor neurons (Cattaert et al., 2002; Torkkeli and Panek, 2002). Such modulation facilitates the detection of biologically relevant information by integrating inputs from multiple senses, and may adjust the weight of different sensory systems in the integrative behavior of the animal. For example, visual perception can be altered by sound or touch, enhancing detection of visual signals or sometimes creating visual illusions (Shams and Kim, 2010).

The combination of modulation within and across senses enables sensory perception to be highly adaptive and dynamic. Modulation actually prevents an animal from having an accurate and absolute perception of the environment, but instead allows an animal to extract information relevant to survival from its environment while ignoring biologically-irrelevant stimuli. Therefore, an animal's adaptability to diverse environments depends on the modulation of its sensory system.

Modulation within a particular sense

1. Adaptation

Sensory perception can be modulated by stimuli to the same sense in multiple ways. One simple form of such modulation is adaptation, which reduces sensory transduction when a sustained stimulus is present. Adaptation facilitates the detection of relative changes in stimuli, and increases the dynamic range of a sensory system.

Adaptation allows a system with limited dynamic range to detect small changes in signals in an environment with a much larger dynamic range. One of the best examples is in the vertebrate visual system (Fain et al., 2001). In the vertebrate photoreceptors, activation of the photopigment rhodopsin activates the heterotrimeric G protein

Transducin, which regulates a cGMP phosphodiesterase. The reduction of cGMP closes cyclic nucleotide-gated channels, producing an electrical response. These channels, however, are also permeable to calcium, an important secondary messenger in visual adaptation. When presented with background light, the closing of cyclic nucleotide-gated channels decreases intracellular calcium levels. The reduced calcium level modulates multiple steps in the phototransduction pathway to reduce the gain of the transduction pathway. If background light is strong enough, the light can bleach the photopigment rhodopsin into rhodopsin intermediates, such as Meta II and opsin. These rhodopsin intermediates directly activate the phototransduction pathway independent of illumination and reduce intracellular calcium levels, further lowering sensitivity (Fain et al., 2001). These changes in the phototransduction pathway adjust the limited detection range of the system to fit the dynamic range of the current surroundings, allowing optimal detection of visual information under diverse illumination conditions.

Another role of adaptation is to allow detection of changes in stimuli using a system that detects absolute levels of stimuli. The changes in stimuli are usually more useful to an animal than the absolute strength of the stimuli. This effect is evident in chemosensation in *C. elegans*, where changes in the concentration of attractant modulates a biased random walk (Pierce-Shimomura et al., 1999; Bargmann, 2006). If the animal moves down an attractant gradient, the reduction in attractant concentration increases the turning rate of the animal, reorienting the animal so that it may move up the gradient. The chemosensory cells, such as AWC or ASER cells, show calcium response only when the concentration of an attractant/repellent changes, and adapt to the new concentration in about a minute (Chalasani et al., 2007; Suzuki et al., 2008). Therefore, adaptation in

these cells constantly resets the baseline to recent stimulus levels. By comparing the current level of stimuli to the baseline, the chemosensory system senses changes of concentration over time using receptors that detect absolute concentrations.

2. Habituation

When a stimulus is repeatedly given to an animal, the animal habituates to the stimulus. Unlike in adaptation, however, presenting habituated animal with a new stimulus can restore responses to the old stimulus (dishabituation; Pinsker et al., 1970). This is because that the diminished response is caused not by reduced sensory sensitivity, but by suppression of downstream circuit elements.

For example, repeated stimuli in *Aplysia* attenuates the siphon withdrawal reflex through habituation (Castellucci et al., 1970; Kupfermann et al., 1970). Habituation reduces the strength of synapses, but does not affect the peripheral sensory receptors (Castellucci et al., 1970; Kupfermann et al., 1970; Castellucci et al., 1978). Therefore habituation does not affect sensory transduction, but regulates the downstream transmission of the signal. Similarly, the *C. elegans* chemosensory system also habituates to a certain odor after prolonged exposure while maintaining sensitivity to other odors (Colbert and Bargmann, 1995). These changes in the transmission of the sensory signals shift the weight of different input stimuli on behavior responses, allowing the animal to ignore repeated stimuli that are not associated with useful information.

Sensitization

Although an animal habituates to repeated harmless stimuli, exposure to noxious stimuli enhances withdrawal or escape responses that may be generated even by harmless stimuli. In *Aplysia*, an electrical shock to the animal's mantle enhances contraction of the

siphon (Pinsker et al., 1973) through presynaptic facilitation (Castellucci and Kandel, 1976. The noxious shock stimulus activates interneurons that synapse onto the sensory neurons that initiate the gill-withdrawal reflex. The activation of the facilitating neuron increases cAMP levels in the sensory neuron (Bernier et al., 1982), which activates the cAMP-dependent protein kinase (Castellucci et al., 1982; Shuster et al., 1985) to strengthen the synaptic strength from the sensory neuron to the motor neuron. This effect can last for up to 3 weeks, and the long-term effect requires RNA and protein synthesis (Montarolo et al., 1986).

Sensitization differs from associative learning because the two stimuli do not need to be coupled. Because the obnoxious stimulus in sensitization is sensed by a different sensory system, sensitization is a simple form of modulation in which one type of stimulus modifies the response of the animal to other types of stimuli. However, sensitization can also occur within the same sensory cell in *C. elegans* mechanosensation (Rankin et al., 1990).

Inter-modal sensory modulation

In sensitization, sensory perception can be modulated by obnoxious stimuli. However, sensory perception can also be modulated by non-obnoxious stimuli detected by other senses. For example, different human senses influence one another. For a particular human task, one form of sensory perception is usually dominant, and strongly influences non-dominant forms of sensory perception. When a task involves spatial discrimination, visual perception is usually the dominant sensory perception because it provides high-resolution information. For example, when an observer is asked to determine the shape of an object by simultaneously seeing a distorted image of the object

and touching it, the final impression is strongly dominated by the distorted image that is seen (Rock and Victor, 1964). Similarly, when an observer is asked to locate the source of a sound stimulus accompanied by a flash, the location of the flash strongly influences the perceived location of the sound (Thurlow and Jack, 1973). On the other hand, auditory perception becomes dominant when temporal discrimination is involved: when both a continuous tone and light are presented, the perceived duration of the stimulus is strongly affected by the perceived length of the tone (Walker and Scott, 1981). Sound can sometimes even change the qualitative perception of visual stimuli, creating visual illusions: when a single flash is presented with multiple beeps, the flash is usually perceived as multiple flashes (Shams et al., 2000). The dominance of hearing over vision under these situations is probably because hearing has better temporal resolution than vision (Shams and Kim, 2010).

However, a dominant sense can also be modulated by non-dominant senses (Shams and Kim, 2010; Lalanne and Lorenceau, 2004). For example, speech recognition, a process mainly dominated by hearing, can be modified by visual processing of the movement of the lips (McGurk and MacDonald, 1976). Visual perception can also be modulated by tactile or auditory information. Some of these effects occur at the higher cognitive levels, such as sound-enhanced contrast detection (Lippert et al., 2007, but other modulation may enhance perception through sensory integration before cognition (Frassinetti et al., 2002).

Higher cognitive processes involving visual perception can also be modulated by stimuli from other senses. For example, visual perception of shape or distance can be calibrated to adapt to new environments. When information about the shape or distance

obtained by visual perception is inconsistent with the shape or distance as sensed by touch, visual perception is recalibrated by tactile information (Adams et al., 2004; Atkins et al., 2003). In addition, visual learning and memory can also be enhanced by paired sound stimuli (Murray et al., 2004). Similar facilitation between olfactory and visual learning has also been observed in invertebrates like the fruit fly (Guo and Guo, 2005).

Such modulation usually involves higher-level neurons instead of the sensory neurons themselves, especially in human where complex interneuron network is responsible for perception and cognition. Indeed, Meredith and Stein (1983) have shown that neurons in the superior colliculus in cats respond better when both visual and auditory stimuli are given together, especially if the signals are weak. The exact mechanism of how interneurons integrate signals from multiple senses to modulate perception remains unknown.

Sensory modulation by motor functions

Sensory perception provides environmental information to the animal, which then responds accordingly through the motor system. However, the motor system can also feed back to the sensory system to coordinate behaviors. Such modulation of sensory perception by motor neurons has been well demonstrated in the proprioception of crustaceans. The upward or downward position of a leg in crayfish is sensed by a specialized proprioceptor called Coxo-Basipodite Chordotonal Organ (CBCO) across the second joint of the leg (Cattaert et al., 2002). When the second joint of the leg is bent by outside force, mechanosensory cells in the CBCO active motor neurons controlling the second joint to generate force opposing the outside force. This reaction is called the "resistance reflex". However, the motor neurons and upstream interneurons also synapse

onto these mechanosensory neurons and inhibit their activities through presynaptic inhibition. At least three different synaptic transmitters with different functions are involved in this process: GABA, histamine and glutamate. GABAergic innervation of the CBCO maintains a low-level inhibition in the absence of rhythmic movements, allowing fine-tuning of the gain of the resistance reflex. When the animal is moving rhythmically, however, both GABA and histamine induce strong inhibition to block mechanosensory transduction (Cattaert et al., 1992; El Manira et al., 1991). In contrast, glutamate only induces small and slow-developing depolarization in the CBCO. Such depolarization correlates directly with motor neuron activities, and therefore may act as a gain-control mechanism for the motor neurons (Cattaert and Le Ray, 1998). The combined effects of presynaptic inhibition coordinates motor programs and ensures that the CBCO does not trigger resistance reflexes during movements.

Actions in vertebrates also affect perception. In human, for example, pressing a left or right arrow key interferes with identification of an image of an arrow of the same direction (Musseler and Hommel, 1997). Similarly, performing hand actions enhances the discrimination of hand posture images (Miall et al., 2006). These processes, however, likely involve higher cognitive function. The exact mechanism underlying these processes is unclear.

Neuropeptide modulation

As discussed above, many neurons are modulated through synaptic transmission. However, some neuronal modulation occurs through non-synaptic neuropeptides (van den Pol, 2012). Neuropeptides can be secreted from synaptic or non-synaptic regions of a neuron (Morris and Pow, 1991) and binds receptors on nearby or distant targets. The

targets of neuropeptides can be pre- or post-synaptic to its origin (Chalasani et al., 2010), or not directly connected with its origin (Pocock and Hobert, 2010). Neuropeptides can also modulate large numbers of long-distance targets within the brain through volume transmission (Fuxe et al., 2007).

Several examples of neuropeptide modulation occur in invertebrates such as *C. elegans* and *Drosophila*, whose neuronal networks are much simpler than vertebrates. In the *C. elegans* chemosensory system, two neuropeptides form a feedback inhibition loop between a sensory neuron and its downstream interneuron. The chemosensory AWC neurons repress AIA interneurons (Chalasani et al., 2010) through glutamate and neuropeptide NLP-1. NLP-1 activates NPR-11, a G protein-coupled receptor expressed in AIA, and initiate feedback from AIA, which releases INS-1, an insulin-like peptide, back to AWC. INS-1 then inhibits AWC activity through an unknown receptor, dampening the activity of the AWC cells to adapt to a constant level of odor. Similar feedback inhibitions has been observed in the *Drosophila* olfactory system (Ignell et al., 2009). In a separate example in the *C. elegans* gustatory system, a neuropeptide, FLP-21, activates a latent circuit for gustatory chemosensation under hypoxic conditions. Two pairs of sensory neurons, ADF and ASG, contribute to gustatory behavior significantly only under hypoxic conditions, not under normal conditions (Pocock and Hobert, 2010). Under hypoxic conditions, ADF and ASG secrete serotonin that activates the M4 pharyngeal motorneurons, which is not connected directly to the ADF or ASG cells. The M4 cell then initiates feedback by secreting the neuropeptide FLP-21, which acts on AQR, PQR and URX cells to enhance their gustatory functions under hypoxic conditions.

These examples demonstrate that neuropeptide modulation has profound impact on sensory function.

In addition to modulating sensory perception, neuropeptides also modulate higher cognitive functions such as learning. In *C. elegans*, mutations in genes encoding an insulin-like peptide (*ins-1*), the insulin receptor (*daf-2*), or downstream kinases including PI3k (*age-1*), PDK (*pdk-1*) and AKT (*akt-1*) all lead to decreased memory acquisition and retrieval (Tomioka et al., 2006; Kodama et al., 2006; Lin et al., 2010). Similarly, an insulin-like hormone, IGF2, is crucial for memory consolidation in inhibitory avoidance learning in mice (Chen et al., 2011). These types of modulation complement classical synaptic transmission and provide extra plasticity in the nervous system.

Hormonal modulation of sensory perception

Sensory systems can also be modulated by hormones secreted by distant sources. These hormones provide additional input from non-neuronal peripheral systems, such as the reproductive system and the digestive system, to the sensory systems. For example, songbirds respond differently to bird songs during the mating season and non-mating seasons (Maney and Pinaud, 2011). Such changes depend on elevated levels of estradiol during the mating season, which modulates auditory responses to bird songs (Tremere and Pinaud, 2011). Similarly, female gray treefrogs prefer male advertising calls more when injected with progesterone and prostaglandin (Gordon and Gerhardt, 2009).

Hormonal modulation are not limited to sex hormones. Several mammalian hormones, including but not limited to insulin, ghrelin and leptin, signal blood glucose levels and energy balance of the body to the nervous system. These hormones modulate olfaction by acting in either the olfactory bulb or the olfactory mucosa (Palouzier-

Paulignan et al., 2012) to attenuate olfactory attraction to food when the energy level is high. In addition, insulin also increases pain threshold in mice (Rajendran et al., 2001). These examples suggest significant roles of hormonal modulation in sensory perception. In simple invertebrates like *C. elegans*, hormonal modulation and long-range neuropeptide modulation are virtually the same because of the lack of a circulation system.

Mechanosensation and its modulation

Force sensing is the underlying mechanism for several sensory modalities, including hearing, touch/pain sensation and proprioception. Each mechanosensory system employs highly specialized structures suitable for detecting specific kinds of mechanical stimuli in a wide range of situations. The proper function of these mechanosensory systems depend on modulation.

For example, the vertebrate auditory system detects weak signals in ambient background noise that varies over 120 dB using a single sensory system that is highly modulated. In the inner ear, the organ of Corti contains four rows of hair cells that mediate hearing (Schwander et al., 2010). Three rows of outer hair cells (OHCs) actively amplify the signal and one row of inner hair cells (IHCs) transduces mechanical signals into electrical signals. Each hair cell has several dozen stereocilia of varying lengths. The tip of each stereocilia contains mechanosensitive channels (Beurg et al., 2009), and is connected to the side of a nearby longer stereocilium through tip links essential for mechanosensation (Assad et al., 1991; Zhao et al., 1996). Two cadherins (cadherin 23 and protocadherin 15) form the tip link (Downey et al., 2006; Kazmierczak et al., 2007; Siemens et al., 2004). The tip link is held on the side of the longer stereocilium by MYO1c (Steyger et al., 1998), a myosin motor that maintains tension in the tip links. The mechanotransduction channel has not been identified, but recent evidence suggests that two redundant channel-like proteins, TMC1 and TMC2, may be part of the mechanotransduction complex (Kawashima et al., 2011).

Several mechanisms enable the hair cells to sense a large dynamic range. The amplification mediated by the OHCs is non-linear, resulting in larger amplification when

the stimulus is small and smaller amplification when the stimulus is large. Such non-linearity in the amplification mechanism compresses over 120 dB differences in input signal into only 20 dB differences in mechanotransduction output (Hudspeth, 2008; Robles and Ruggero, 2001). In addition to the non-linear amplification, adaptation in the hair cells maintains sensitivity to changes in sound pressure under continuous stimulation. The mechanotransduction channel partially adapts to sustained deflection by two mechanisms. Calcium entering through the opening of the channel reduces the opening probability of the channel in respond to a certain deflection (Schwander et al., 2010). Such adaptation occurs within millisecond, but saturates quickly (Stauffer et al., 2005). This fast adaptation is then accompanied by a slower adaptation caused by relaxing the tension on the mechanotransduction channel. Deflection of the stereocilia increases tension in the tip link, which causes MYO1c, the myosin motor that actively maintains tension in the tip link, to slip. Blocking MYO1c activity was first shown to inhibit slow adaptation (Holt et al., 2002), but later studies showed that it also affects fast adaptation (Stauffer et al., 2005). The combined effect of non-linear amplification and adaptation enables the hair cells to function under highly dynamic background levels while maintaining sensitivity to weak signals.

The mammalian skin distinguishes different types of forces using a combination of specialized receptors with different adaptation rates. For example, the Pacinian corpuscle adapts quickly, and therefore senses vibration and distinguishes textual information; the Merkel cells, however, adapts slowly, and encode information about edges and shapes. Stronger stimuli activate the C-fibers and nociceptors, which encode pain. The combination of information gathered by these different touch receptors forms

our sense of touch. The different adaptation characteristics of touch receptors are essential in mammalian mechanosensation.

The significant roles of modulation in hearing suggests that the touch sensation system may also have an elaborate modulatory system to cope with conditions with changing background forces, as in the auditory system. Indeed, dysfunctional modulation of touch/pain sensitivity causes pathological conditions such as hyperalgesia and hyperallodynia. These conditions are usually caused by non-mechanical factors, such as inflammation (Dina et al., 2004). Some cellular components have been associated with these conditions, such as integrin signaling and TRP channels (Dina et al., 2004; Fujii et al., 2008; Ro et al., 2009). However, the full molecular mechanisms underlying modulation of touch sensitivity remains unknown.

Mechanosensation in *C. elegans*

C. elegans responds to a mechanical stimulus by moving away from the source of the stimulus. When the anterior end of the animal is touched, it moves backward; when the posterior end of the animal is touched, it moves forward. Like the touch receptors in mammalian skin, *C. elegans* senses different mechanical signals with different groups of mechanosensitive neurons, depending on the strength and the position of the mechanical stimuli.

Harsh touch along the body is sensed by three groups of neurons: BDU, SDQR, FLP, ADE and AQR neurons for anterior touch, PVD and PDE cells for posterior touch, and PHA/PHB cells for harsh touch on the anus (Li et al., 2011). The anterior cells signal through the interneurons AVA, AVD and AVE to mediate backward movement, and the posterior cells require the interneurons PVC and DVA for forward movement. Most of

these sensory cells synapse onto the downstream interneurons through chemical synapses (White et al., 1986), though the exact connections required for harsh touch response are unknown.

Touch to the tip of the nose is sensed by three classes of ciliated neurons near the head of the animal, the ASH, FLP and OLQ cells (Kaplan and Horvitz, 1993). The mechanosensory functions of these cells require intact cilia. In addition to sensing mechanical signals, the ASH cells also sense chemical and osmotic stimuli through different signaling pathways, thus acting as multi-modal proprioceptors (Kaplan and Horvitz, 1993; Hart et al., 1999; Hilliard et al., 2005; Sambongi et al., 1999). Two TRPV channels, OSM-9 and OCR-2, are required for mechanosensation, chemosensation and osmosensation in ASH cells (Colbert et al., 1997; Tobin et al., 2002). These channels, however, are not required for the initial mechanotransduction in ASH neurons. Mechanotransduction in these cells require the DEG/ENaC channel DEG-1 and possibly another unidentified channel (Geffeney et al., 2011).

Gentle touch to the side of the body is sensed by six touch receptor neurons (TRNs) (Chalfie and Thomson, 1979; Chalfie and Sulston, 1981). Laser-ablation experiments showed that three anterior cells (ALML/R and AVM) sense touch to the anterior half of the body, and two posterior cells (PLML/R) sense touch to the posterior half of the body. The PVM cell does not contribute to touch sensation significantly, and also has slightly different gene expression profiles from the other five TRNs (Chalfie and Sulston, 1981; Topalidou et al., 2011). Saturated mutagenesis screens and subsequent microarray analyses have identified a number of genes required for mechanotransduction in these cells (Chalfie and Au, 1989; Zhang et al., 2002; Topalidou and Chalfie, 2011),

including genes that encode the core mechanotransduction complex, which is composed of the DEG/ENaC channel subunits (encoded by *mec-4* and *mec-10*; O'Hagan et al., 2005; Arnadottir et al., 2011) and auxiliary proteins (encoded by *mec-2* and *mec-6*; Chelur et al., 2002; Goodman et al., 2002; Zhang et al., 2004), genes that encode extracellular matrix (ECM) proteins (*mec-1*, *mec-5* and *mec-9*; Emtage et al., 2004), cytoskeleton proteins (*mec-7*, *mec-12* and *mec-17*; Savage et al., 1989; Fukushige et al., 1999; Akella et al., 2010), and several other genes (*mec-8* encoding a splicing factor required for *mec-2* splicing, *mec-14* encoding a homolog of the β subunit of Shaker-type potassium channels, *mec-15* encoding an F-box protein, and *mec-18* encoding a firefly luciferase homolog; Spike et al., 2002; Calixto et al., 2010b; Bounoutas et al., 2009b). Two other genes, *unc-86* and *mec-3*, encode transcription factors needed to specify TRN cell fates (Chalfie and Sulston, 1981; Finney et al., 1988; Way and Chalfie, 1988; Way and Chalfie, 1989). Studies in the last two decades have elucidated how the products of some of these genes interact and function in mechanotransduction.

Stimulation of the TRNs activates or inhibits downstream command interneurons, the AVA, AVB, AVD and PVC cells (Figure 1; Chalfie et al., 1985). These cells then direct the movement of the animal: AVA and AVD cells mediate backward movement, and AVB and PVC cells mediate forward movement. Activation of the anterior TRNs (two ALM and one AVM cells) promotes backward movement by activating the AVD interneuron through gap junctions and inhibiting the AVB and PVC interneurons through chemical synapses (Chalfie et al., 1985). The connection to the AVB cell is established late in development through the AVM cell (Chalfie et al., 1985). Activation of the posterior TRNs promotes forward movement by activating the PVC interneuron through

gap junctions and inhibiting the AVA and AVD interneurons through glutamatergic synapses. Therefore, all major excitatory pathways utilize gap junctions instead of chemical synapses, which are inhibitory (Chalfie et al., 1985). This high dependence on gap junctions is uncommon among many other neuronal circuits known in *C. elegans*, including chemosensation, oxygen-sensation, motor controls, and the harsh touch circuit described above (Gray et al., 2005; Chalasani et al., 2007; Macosko et al., 2009). Although the functional circuits for these pathways are not all known, these circuits utilize mostly chemical synapses between the sensory cells and the interneurons, as judged from existing anatomical data (White et al., 1986). The gap junctions in the touch response circuit are not known to be modified through synaptic modulation, such as classical presynaptic inhibition and facilitation, but are more robust (Kandel et al., 2000). The quickness and robustness of the touch response is crucial for the survival. Slower movement in response to touch, for example, reduces the chance of escaping from predatory fungi (Maguire et al., 2011).

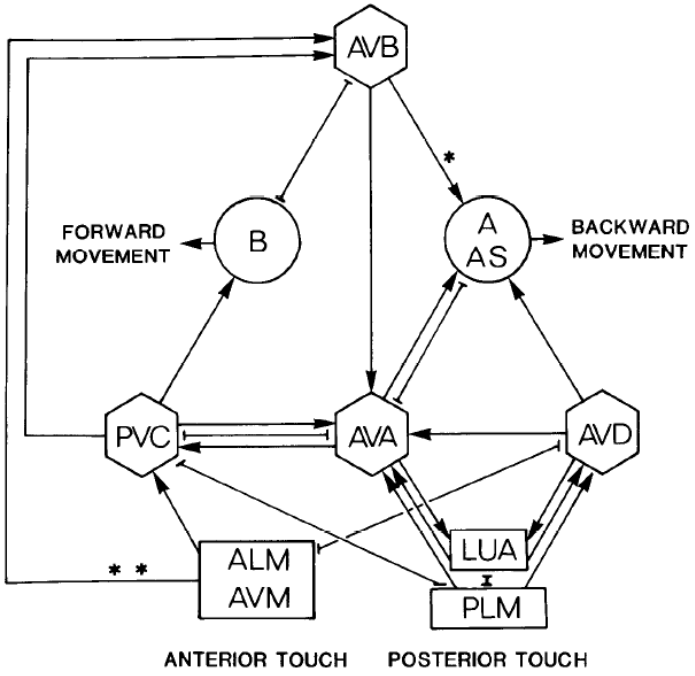

Figure 1. The gentle touch circuit. Arrows indicate chemical synapses and flat arrows indicate gap junctions. *AVB only synapses onto the AS cells, not the A cells. **Only the AVM cell, but not the ALM cells forms chemical synapse with AVB. Reprinted from Chalfie et al., 1985.

The genetics of mechanotransduction in the TRNs

Mechanotransduction in the TRNs requires a DEG/ENaC channel composed of two channel subunits, MEC-4 and MEC-10, and two auxiliary proteins, MEC-2 and MEC-6. These four proteins co-localize in the TRN processes, and co-precipitate when expressed in a heterologous system (Chelur et al., 2002; Zhang et al., 2004; Arnadottir et al., 2011). When MEC-2 and MEC-6 are expressed in *Xenopus* oocytes with a mutant MEC-4 that is constitutively open (MEC-4(d)), the current produced by MEC-4(d) is greatly increased (Chelur et al., 2002; Goodman et al., 2002). Furthermore, TRNs lacking MEC-2, MEC-4 or MEC-6 do not produce mechanoreceptor currents (Chelur et

al., 2002; Goodman et al., 2002; O'Hagan et al., 2005), suggesting that these three proteins are essential for mechanotransduction. MEC-10 is a modulatory unit for the mechanotransduction channel, because animals lacking MEC-10 are only partially touch-sensitive and produce mechanoreceptor currents smaller than wild-type animals (Arnadottir et al., 2011). Finally, the TRNs in animals containing either of two mutations, *mec-4(u2)* or *mec-10(u20)*, have different reversal potentials for the mechanoreceptor current and a longer latency for channel opening, suggesting that these two mutations affect the ion selectivity and gating of the channel (O'Hagan et al., 2005). These results demonstrate that MEC-4 and MEC-10 form the mechanotransduction channel, and both MEC-2 and MEC-6 are required for the function of the MEC-4/MEC-10 channel.

In addition to expressing the channel components needed for mechanosensation, the TRNs have specialized structures that are physically well suited for sensing gentle touch along the body. These cells have processes that run along the length of the body embedded in the hypodermis (Chalfie and Thomson, 1979). The processes are filled with 15-protofilament microtubules, which are present only in these six cells in *C. elegans* (Chalfie and Sulston, 1981). The 15-protofilament microtubules are formed by two tubulins, MEC-7 and MEC-12, expressed specifically in the TRNs (Fukushige et al., 1999; Savage et al., 1989; Savage et al., 1994; Hamelin et al., 1992). These specialized microtubules also require acetylation by the microtubule acetyltransferase MEC-17 to stabilize the structures (Akella et al., 2010; Cueva et al., 2012; Shida et al., 2010; Topalidou et al., 2012). These microtubules are oriented in the same direction with one end in the center of the process and the other end at the periphery (Chalfie and Sulston, 1981). The presence of microtubules is essential for proper axonal growth and gene

expression in the TRNs, but the special 15-protofilaments are not essential for these functions (Bounoutas et al., 2009a; Fukushige et al., 1999; Savage et al., 1989; Bounoutas et al., 2011; Savage et al., 1994; Topalidou and Chalfie, 2011). *mec-7* and *mec-12* mutants still showed residual, albeit much reduced mechanoreceptor currents (Bounoutas et al., 2009a), further indicating that the 15-protofilament microtubules are not essential for mechanotransduction. However, the 15-protofilaments may provide extra mechanical strength to support mechanotransductions, because the wild-type MEC-17, which catalyzes the acetylation of microbutules in the TRNs and facilitates the formation of the 15-protofilament microtubules, is more effective in restoring touch sensitivity in *mec-17* mutants than catalytically inactive forms of MEC-17, which does not restore the 15-protofilament microtubules (Shida et al., 2010; Topalidou et al., 2012).

The TRN processes are attached to the hypodermis through hemi-desomosome like structures (Chalfie and Sulston, 1981). These attachment points anchor the TRNs to the hypodermis, and separate the process from the body-wall muscle during development. Mutations in genes affecting the extracellular matrix, including *him-4* (hemicentin), *mec-5* (collagen), *mec-1* and *mec-9* (EGF/Kunitz domain containing proteins), all disrupt the attachment between the TRNs and the hypodermis, causing the process to appear adjacent to the body-wall muscle in adult animals (Vogel and Hedgecock, 2001; Emtage et al., 2004). Although mutations in *mec-5*, *mec-1* and *mec-9* cause loss of touch sensitivity (Chalfie and Au, 1989), *him-4* animals still have residual touch sensitivity (Vogel and Hedgecock, 2001), suggesting that the attachment is not essential for mechanotransduction. Nevertheless, the attachments may contribute to touch sensitivity

by anchoring the TRNs at the optimal physical position and physically facilitating force transmission.

Therefore, both the cytoskeletal and ECM components of the TRNs play important roles in mechanosensation. Although neither component is absolutely required for mechanotransduction like the mechanotransduction channel components, the cytoskeletal and ECM components provide the TRNs with the required physical basis for efficiently transducing mechanical force.

Integrin signaling in mechanosensation

The body-wall muscle is another mechanotransductive tissue in *C. elegans* that functions in reverse to the TRNs: TRNs detect mechanical forces and transduce them into electrical signals, but the body-wall muscles transduce electrical neuronal signals into mechanical forces. Surprisingly, these two distinctly different tissues share certain structural and molecular components. The body-wall muscle is also anchored to the hypodermis through hemi-demosome like attachments called Fibrous Organelle (Francis and Waterston, 1991). Unlike the TRN attachments, however, these muscle attachments are crucial for normal muscle functions because they transmit the force generated in the TRNs to the hypodermis, thus inducing movements. Force transmission from the body-wall muscle to the hypodermis requires the dense bodies, a specialized attachment complex that anchors the actin and myosin filaments in the muscle to the cell membrane. The dense body consists of the α and β integrins (PAT-2/PAT-3), Mig-2 (UNC-112), Integrin-linked kinase (PAT-4), PINCH (UNC-97), talin (Y71G12B.11), vinculin (DEB-1) and other proteins (Moerman and Williams, 2006). Loss-of-function mutations in

genes encoding many of these proteins cause a Pat phenotype (paralyzed arrested elongation two fold) because of muscle function failure (Williams and Waterston, 1994).

Many integrin signaling genes, including all of the dense-body genes mentioned above, are conserved across species (Zaidel-Bar, 2009). The mammalian homologs of the dense body proteins form focal adhesion complexes that physically link the extracellular matrix with the actin cytoskeleton. Disrupting these genes either biochemically or genetically causes defects in cellular shape, motility, the formation of the ECM and cell proliferation (Persad et al., 2000; Cruet-Hennequart et al., 2003; Zhang et al., 2002; Guo and Wu, 2002). In the fruit fly, disrupting these genes causes lethality and defects in embryonic muscle attachment and dorsal closure (Brown, 1994; Clark et al., 2003; Zervas et al., 2001). Similarly, disrupting the mouse homologs causes embryonic lethality (Fässler and Meyer, 1995; Stephens et al., 1995; Sakai et al., 2003; Li et al., 2005) and an increase in endodermal-cell apoptotsis (Li et al., 2005). In addition, mutations in the mammalian homolog of UNC-112, kindlin-1, causes Kindler syndrome (Siegel et al., 2003). These observations suggest important roles of the focal adhesion proteins in animal developments.

The focal adhesion proteins also sense forces exerted on the cell through adhesions (Chen, 2008). Stretching forces reinforce cell adhesion (Roca-Cusachs et al., 2009) and induce cellular changes (Vogel and Sheetz, 2009) through integrin signaling. Cell-adhesion mediated mechanotransduction, however, is different from mechanotransduction in the TRNs. Integrin-mediated mechanotransduction is much slower (occurs over tens of seconds; Vogel and Sheetz, 2009) than neuronal mechanotransduction in the TRNs, fly bristles, vertebrate hair cells, and Pacinian

corpuscles (occurs within ~1 msec; O'Hagan et al., 2005; Corey and Hudspeth, 1979; Walker et al., 2000; Gray and Malcolm, 1950). This is because integrin-mediated mechanotransduction requires chemical signaling cascades and phosphorylation (Geiger and Bershadsky, 2001; Bershadsky et al., 2006), which are much slower than the directly mechanosensitive channels utilized in mechanosensory neurons.

Many focal adhesion genes, including *pat-2*, *pat-4*, *pat-6* and *unc-97*, are also expressed in the TRNs (Gettner et al., 1995; Mackinnon et al., 2002; Lin et al., 2003; Hobert et al., 1999). Animals with reduced *unc-97* activity are touch insensitive in a sensitized background (Hobert et al., 1999), suggesting that the focal adhesion proteins may affect mechanosensation. However, the exact roles of these proteins in the TRN are hard to study because loss-of-function mutations of these genes produce lethality, making it impossible to test touch sensitivity directly. Whether the focal adhesion proteins function as mechanical tethers, as in the body-wall muscles, or as signaling molecules, as in the focal adhesions, is unclear.

Habituation and sensitization in the TRNs

Modulation of the TRNs has been studied using an animal's response to plate tapping, which activates both the anterior and posterior TRNs (Rankin et al., 1990; Chiba and Rankin, 1990). These studies have shown that the tap response displays plasticity similar to that seen in the *Aplysia* siphon-withdrawal reflex (Rankin et al., 1990), including habituation, dis-habituation and sensitization.

Repeated taps habituate TRNs and reduce the strength of the response to subsequent taps (Rankin et al., 1990). This effect can be reverted by an electrical shock across the animal, similar to dis-habituation observed in *Aplysia*. In addition, the tap

response also increases in intensity after a strong tap (Rankin et al., 1990), showing that the TRN response can be sensitized. Such modulation occurs pre-synaptic to the command interneurons, because repeated taps do not reduce the magnitude of spontaneous reversals or reversals caused by other stimuli that are also transduced through the command interneurons (Wicks and Rankin, 1997). These results suggest that habituation may occur at or upstream of the synapses from the TRNs to the interneurons.

In support of this hypothesis, mutations in *eat-4*, which encodes the vesicular glutamate transporter (Lee et al., 1999; Reimer et al., 2001), enhance habituation and inhibit recovery from habituation (Rankin and Wicks, 2000). However, null mutations in *glr-1*, which encodes a glutamate receptor expressed in the command interneurons (Hart et al., 1995; Maricq et al., 1995), eliminate only long-term habituation, not short-term habituation (Rose et al., 2003). In addition, *eat-4* animals, which are deficient in glutamatergic neurotransmission, still respond to anterior touch, suggesting that the gap junctions are sufficient to relay the touch reflex (Lee et al., 1999). The partial effects of *glr-1* mutations on habituation and the non-essentiality of glutamatergic synapses demonstrate that habituation may occur partially independent of the chemical synapses.

Indeed, habituation also occurs upstream of chemical synapses in the TRNs. Although mechanoreceptor currents in the TRNs do not habituate after repeated stimuli (O'Hagan et al., 2005), calcium responses elicited by touch decrease in amplitude following repeated stimuli (Suzuki et al., 2003), suggesting that the signal transmission downstream of mechanotransduction, but upstream of synaptic transmission, may be affected by habituation.

Habituation of the calcium response in the TRNs can be enhanced either by intracellular calcium (Kindt et al., 2007) or by extracellular dopamine produced in other neurons (Sanyal et al., 2004). Dopamine is produced in CEP mechanosensory neurons that detect the texture of food, and modulates habituation in the TRNs depending on the presence of food (Kindt et al., 2007)). Because the CEP neurons do not directly synapse onto the ALM neurons, dopamine released from the CEP neurons modulates responses to mechanosensory stimuli by modulating the rate of habituation through non-synaptic long-range modulation (Kindt et al., 2007).

The tap response can also be sensitized by training (Rankin et al., 1990; Ebrahimi and Rankin, 2007). Two kinds of sensitization have been described. The response to tap increases in magnitude either after a single strong stimulus (Rankin et al., 1990), or after several trains of stimuli at the L1 stage (Ebrahimi and Rankin, 2007). Conversely, if animals are deprived of mechanosensory stimuli during development by being raised isolated, the amplitudes of their responses to tap are reduced (Rose et al., 2005). Sensory deprivation affects the expression of GLR-1 and a synaptic marker SNB-1 (Rai and Rankin, 2007; Ebrahimi and Rankin, 2007), suggesting that sensitization also involves modulating the inhibitory glutamatergic synapses.

These examples demonstrate that the touch response of the TRNs can be modulated by previous experience or external stimuli. Although both habituation and sensitization appear behaviorally similar to the modulation of the siphon withdrawal reflex in *Aplysia* (Pinsker et al., 1970; Pinsker et al., 1973), the mechanisms may differ from those in *Aplysia* because of the major contribution of gap junctions in the TRN touch circuit. Although some evidence suggests that the glutamatergic synapses between

the TRNs and downstream interneurons are modulated by habituation and sensitization, the touch response itself mostly depends on the gap junctions, suggesting that modulation of transduction within the TRNs may be as important as modulation of the chemical synapses. Calcium imaging in the TRNs during repeated stimuli supports this hypothesis. However, how touch sensitivity is modulated, and what factors modulate mechanotransduction, remain unclear.

Insulin signaling and dauer

One of the major conserved signaling pathways that interact the focal adhesion proteins is insulin signaling (Zaidel-Bar, 2009). In *C. elegans*, insulin signaling was first studied in its role in dauer arrest. The dauer stage is an alternative larval stage under stress conditions. Instead of developing from the L1 stage to L2, L3 and then L4 stage, animals under stress conditions can develop into an alternative L2d stage, and then into dauer larvae, at which point their developments are arrested. If conditions improve, they will exit the dauer stage and resume development into L4 larvae. Dauer larvae have reduced transcription in general, but have increased expression of stress-related genes (Dalley and Golomb, 1992). Their metabolism is also different from L3 animals: while animals grown under normal conditions switch from anabolic to aerobic respiration when they grow into the L2 stage, dauer animals do not switch.

Many factors contribute to dauer formation, including a constitutively synthesized dauer pheromone (Golden and Riddle, 1982), the availability of food (Golden and Riddle, 1982), and other environmental conditions such as temperature (Golden and Riddle, 1984ab). Consistent with the multiple controls of dauer formation, several signaling pathways affect dauer formation, including the guanylyl cyclase pathway (*daf-11*, *tax-2*

and *tax-4*; Thomas et al., 1993; Birnby et al., 2000; Coburn et al., 1998), TGFβ-like pathway (*daf-1*, *daf-3*, *daf-4*, *daf-5*, *daf-7*, *daf-8* and *daf-14*; for review, see Patterson and Padgett, 2000; Hu, 2007), insulin-like pathway (*daf-2*, *age-1*, *pdk-1*, *akt-1*, *akt-2*, *daf-16*, *daf-18* and *daf-28*; for review, see Hu, 2007), and a steroid hormone pathway (*daf-9* and *daf-12*; Riddle et al., 1981; Albert and Riddle, 1988; Thomas et al., 1993). The first three pathways act in parallel and upstream of the steroid hormone pathway (Thomas et al., 1993), but some evidence suggests that the guanylyl cyclase pathway may also function upstream of both TGF-like pathway and insulin-like pathway by regulating the expression of *daf-7* and *daf-28* (Murakami et al., 2001; Li et al., 2003). In insulin-like pathway, activation of the DAF-2 insulin receptor initiates a signaling cascade through AGE-1 PI3 kinase, PDK-1 3-phosphoinositide dependent kinase, AKT-1 and AKT-2 protein kinase Bs to inhibit the DAF-16 FoxO transcription factor (Kimura et al., 1997; Morris et al., 1996; Paradis et al., 1999; Paradis and Ruvkun, 1998; Hertweck et al., 2004; Lee et al., 2001; Lin et al., 1997; Lin et al., 2001; Ogg et al., 1997). However, the DAF-2 insulin receptor can also modify DAF-16 activity independent of this pathway (Inoue and Thomas, 2000; Paradis et al., 1999; Paradis and Ruvkun, 1998).

The DAF-2 insulin receptor can be activated by insulin-like peptides. The *C. elegans* genome contains 40 insulin-like peptide genes (*ins-1* to *39* and *daf-28*), but only a few of them contribute to dauer formation. DAF-28, INS-4 and INS-6 promotes insulin-like signaling (Murphy et al., 2003; Li et al., 2003; Cornils et al., 2011), but they function differently in dauer formation: DAF-28 mainly prevents dauer entry, and INS-6 promotes dauer exit (Cornils et al., 2011). INS-1 and INS-18 antagonize insulin signaling to promote dauer entry. The other insulin-like peptides do not contribute

significantly to dauer formation, but may be involved in other functions (See Chapter III introduction for details).

Summary

Mechanosensation functions in diverse tasks, from sensing light strokes and experiencing pleasure to sensing pain and avoiding danger. The touch reflex in the TRNs of *C. elegans* increases chances of escaping mechanical dangers. The touch response itself is graded: a weak tap induces pausing and/or a short backward movement, after which the animal resumes normal movement in its original direction; a stronger tap, however, induces a longer backward movement which is usually followed by an omega turn, leading to movement in a different direction (usually in the opposite direction) (Rankin et al., 1990). Responding incorrectly to a stimulus is either costly, if a non-harmful stimulus induces an omega turn, disrupting an animal's current movement, or dangerous, if a harmful stimulus fails to induce an omega turn to escape. To function under diverse environments and respond correctly to different stimuli, the touch response may be modulated to fit the current environment. Understanding how mechanosensation is modulated, therefore, is crucial to understanding how sensory systems cope with diverse environments.

Traditional forward genetic screens, however, have proven ineffective in studying mechanosensory modulation. Although saturated forward genetic screens have identified multiple genes essential for mechanotransduction or the development of the TRNs, no major modulatory pathway has been revealed. The failure of genetic screens to identify modulatory components in mechanotransduction may implicate several factors: (1) mutations in genes encoding modulatory components of mechanotransduction may cause pleiotropic effects (such as lethality), thus masking the loss of touch sensitivity; (2) these

modulatory components may act redundantly; (3) loss of certain modulatory components may increase touch sensitivity instead of reducing it; (4) loss of modulatory components may only reduce touch sensitivity weakly. Mutagenesis screens would have missed genes with the above properties.

Recent advances in the study of systemic RNA interference (RNAi) provided new tools for tackling these problems. Expressing a double-stranded RNA construct in *E. coli* and feeding the *E.coli* to worms induce systemic RNAi (Fire et al., 1998; Timmons et al., 2001). The dsRNA expressed in the *E.coli* is transported through the gut by SID-2 (Winston et al., 2007), and then into other cells by a dsRNA transporter SID-1 (Winston et al., 2002; Feinberg and Hunter, 2003; Shih and Hunter, 2011). SID-1 expression in the nervous system is low, rendering systemic RNAi in the nervous system ineffective (Winston et al., 2002). By expressing SID-1 only in specific cells or tissues (such as the nervous system) in a *sid-1* null background, our lab has developed a method to enhance feeding RNAi efficiency in the nervous system and knock down gene expression in specific cells (Calixto et al., 2010a). It is therefore possible to look at the effect of knocking down pleiotropic genes in the TRNs without inducing pleiotropic phenotypes.

In this thesis, I attempt to dissect mechanosensory modulation by initially identifying pleiotropic genes that modulate touch sensitivity. I then analyze factors that modulate touch sensitivity, and associate these factors with signaling pathways identified. In Chapter II, I circumvent the lethality associated with pleiotropic genes by tissue-specific feeding RNAi, and identify potential regulatory pathways of mechanosensation, including the integrins and focal adhesion genes. In Chapter III, I focus on mechanosensory modulation by mechanical conditions, and identify an integrin-

dependent long-term sensitization induced by sustained background vibration that counteracts the effect of habituation on touch sensitivity. During such sensitization, the integrins act as secondary mechanosensors in the TRNs independent of the MEC-4 mechanotransduction channels. I also further analyze the roles of integrin signaling in mechanosensory modulation, and identify additional modulatory pathways that may interact with the focal adhesion genes. One of such pathways, insulin signaling, converges with integrin signaling on AKT-1. Finally, I show that both integrin and insulin signaling convergently modulate touch sensitivity thorough ubiquitination, which regulates MEC-4 surface expression. In Chapter IV, I further explore the role of insulin signaling in mechanosensory modulation by identifying insulin-like peptides that modulate touch sensitivity. One of these peptides, INS-10, affects touch sensitivity through long-range neuropeptide modulation. INS-10 transmits stress signals sensed by other neurons to the TRNs and reduces anterior touch sensitivity under diverse stressful conditions. Reducing touch sensitivity increases an animal's efficiency for other tasks under mechanical distractions, thus increasing its chance of survival under stress conditions. In Chapter V, I summarize the patterns of modulation that emerge from the previous two chapters, showing that intra-modal modulation by the mechanical environment and inter-modal modulation by other conditions originate from specialized neurons: long-distance neuropeptide modulation plays important roles in modulation by non-mechanical signals, and a secondary mechanosensory system within the TRNs mediates modulation by mechanical signals sensed directly by the TRNs. All modulation described either enhances stimulus detection, or de-prioritizes mechanosensation to increase efficiency, depending on the need of the animal. I then conclude that the

integration of modulation by multiple mechanical and non-mechanical conditions optimizes mechanosensation under diverse conditions, thus maximizing an animal's ability to adapt to its environments.

Chapter II. Identification of pleiotropic genes affecting touch sensitivity.

(Part of the work described in this chapter, including Figure 2, is published in the paper *Enhanced neuronal RNAi in C. elegans using SID-1*(Calixto et al., 2010a), which is included in Appendix I. The method presented in this paper was developed by A. C., D. C., I. T. and M. C. I used the method to perform experiments described in this chapter, and the results of the focal adhesion genes were included in the paper as Figure 5. Brad Collins, Katie Montelione and Aaron Scheffler helped with the logistics of the RNAi screen.)

Abstract

C. elegans sense gentle touch along the body using six touch receptor neurons (TRNs). Although genetic screens and microarray analyses have accumulated considerable knowledge of how mechanotransduction occurs in the TRNs, no modulatory pathways have been identified using these methods. In this Chapter, I used neuronal-enhanced feeding RNAi to screen genes that cause lethality when mutated, and identified 61 such genes affecting touch sensitivity, including the focal adhesion genes that encode dense body components in the body-wall muscle. RNAi tests of additional focal adhesion and muscle components, and TRN-specific RNAi showed that only the core focal adhesion genes affect touch sensitivity cell-autonomously in the TRN. In addition, I have confirmed six of 12 other genes identified with available alleles. *mca-3*, which encodes a plasma membrane calcium pump, affects the calcium response downstream of mechanotransduction. *tag-170*, a microtubule-associated gene, is needed for microtubule formation specifically in the ALM neurons. These results indicate that many pleiotropic genes are needed for optimal touch sensitivity, and may modulate mechanosensation.

Introduction

The *C. elegans* genome contains more than 19,000 genes. Feeding RNAi against ~86% of these genes, however, only identified 1722 genes whose reduction of expression produced a phenotype (Kamath et al., 2003). Of these 1722 genes, 1170 of them produced non-viable phenotypes. Although the low incidents of phenotype in general can be partially explained by ineffective systemic RNAi in the *C. elegans* nervous system (Kamath et al., 2001), the high number of genes causing lethality when knocked down by RNAi suggests that pleiotropic genes make up a considerable portion of the genome. The functions of these genes are difficult to uncover because of the pleiotropic effects associated with mutating these genes.

In *C. elegans*, gentle touch along the body is sensed by six touch receptor neurons (TRNs) (Goodman, 2006). Saturated mutagenesis screens for touch-insensitive mutants have identified 18 genes required for mechanosensation, including genes encoding components of the mechanotransduction complex, the cytoskeleton, and the extracellular matrix (ECM), and genes affecting the development of the TRNs (Chalfie and Au, 1989; Du and Chalfie, 2001; O'Hagan and Chalfie, 2006; Bounoutas and Chalfie, 2007). Subsequent microarray analyses identifying highly expressed genes in the TRNs have uncovered additional genes needed for TRN function or differentiation (Zhang et al., 2002; Zhang and Chalfie, 2002; Topalidou and Chalfie, 2011; Topalidou et al., 2011). However, both mutageneses and microarrays failed to identify modulatory components of touch sensitivity. One possible cause of such failure is that modulatory components may be pleiotropic. When these genes are mutated in a mutagenesis screen, the mutation would cause other phenotypes that mask touch insensitivity. In addition, because the

TRNs are dispensable for animal survival, these genes must be expressed in non-TRN cells to cause the pleiotropic effects, and thus would not be detected in a microarray that finds TRN-enriched genes.

One example of a pleiotropic gene functioning in the TRNs is *unc-97*. *unc-97* was not identified as a touch sensitivity related gene in any of the previous screens, nor was it identified in either microarray analyses. A partial loss-of-function allele of *unc-97* does not cause touch insensitivity by itself, but reduces the touch sensitivity of animals carrying a temperature-sensitive *mec-3* allele at the permissive temperature (Hobert et al., 1999). *unc-97* encodes a homolog of the mammalian Pinch, which is part of the focal adhesion complex. In *C. elegans*, UNC-97 and other focal adhesion proteins form dense bodies that mechanically link the actin/myosin filaments in the body wall muscle to the extracellular matrix, and transmit the force generated by muscle cells to the cuticle to induce movement (Francis and Waterston, 1985). In addition to UNC-97, the major focal adhesion proteins include the PAT-2 α-integrin and the PAT-3 β-integrin, the integrin-linked kinase PAT-4, the actopaxin PAT-6 and the Mig-2-like protein UNC-112. The majority of these proteins, including PAT-3, PAT-4, PAT-6 and UNC-97, are expressed in the TRNs (Gettner et al., 1995; Mackinnon et al., 2002; Lin et al., 2003; Hobert et al., 1999). Furthermore, the dense bodies co-localize with TRN proteins in the muscle, including MEC-5 (Emtage et al., 2004), MEC-6, and ectopically expressed MEC-4 (Chelur et al., 2002). These observations suggest a role for these proteins in mechanosensation in the TRNs. The study of this role, however, has been hampered by the fact that complete loss of gene activity causes embryonic lethality, Pat (paralyzed, arrested-at-twofold stage) phenotype (Williams and Waterston, 1994).

To study the function of pleiotropic genes in the TRNs, it is necessary to reduce their expression specifically in the TRNs through mosaic analysis or cell-specific RNAi. Mosaic analysis requires rescuing null mutants of the gene and picking animals that have lost the arrays specifically in the TRNs. Such a method is labor-intensive and unsuitable for initial screening. Feeding RNAi, however, can be used to screen large numbers of genes. In *C. elegans*, feeding animals with bacteria expressing dsRNA against genes causes systemic RNAi throughout the body (Timmons et al., 2001). By expressing *sid-1* in all neurons or specifically in the TRNs in animals carrying a *sid-1* null mutation, we were able to restrict the RNAi effect specifically to the nervous system or the TRNs (Calixto et al., 2010a).

In this chapter, I use tissue- and cell-specific feeding RNAi to investigate the roles of pleiotropic genes in TRN functions, and find that 61 lethal genes, including the integrins and focal adhesion proteins, are needed in the TRNs for optimal touch sensitivity. These proteins may modulate mechanosensation in the TRNs.

Results

Pleiotropic genes affect touch sensitivity

In the initial whole genome screens by Fraser et al. (2000) and Kamath et al. (2003), 916 bacterial strains produced lethality or paralysis at any developmental stage. Because of secondary targets, these RNAi bacterial strains knock down the expression of 1011 genes. The final Ahringer RNAi library included 908 of the 916 bacterial strains, corresponding to 1005 genes. To test whether pleiotropic genes affect mechanosensation, we performed RNAi against these 1005 genes in TU3595 and tested anterior touch sensitivity. TU3595 carries a *unc-119p::sid-1*construct that expresses *sid-1* in neurons in a *sid-1; lin-15b* background, and therefore should have enhanced neuronal RNAi phenotype and diminished RNAi phenotype in other tissues when fed with RNAi bacteria. Although the *unc-119* promoter we used also had leaky expression in non-neuronal cells (Calixto et al., 2010a), the lethality or paralysis phenotypes were weak enough in TU3595 to allow testing for touch sensitivity. Knocking down 61 of the 1005 genes reduced touch sensitivity (Table 1). 12 of these genes are involved in general cellular functions such as transcription and translation, and are likely to cause general deficiencies in the TRNs. The remaining 49 genes are involved in protein degradation, calcium signaling, cellular adhesion, cytoskeleton, endo/exocytosis, mitochondria, and signaling pathways such as wnt, hedgehog, small GTPase and MAP kinase. Two of the identified genes, *unc-11* and *unc-97*, have been shown to affect touch sensitivity previously by Chalfie (unpublished data) and Hobert et al. (1999). *tba-1*, which encodes an α tubulin, is likely a false positive, because the same RNAi construct also targets *mec-12* encoding a special α tubulin needed for TRN mechanosensation.

Gene name	Description
Transcription & translation related	
Y47H9C.7	EIF2B β subnit homolog
taf-5	TAF (TBP-associated transcription factor) family
taf-9	TAF (TBP-associated transcription factor) family
nrs-1	asparaginyl-tRNA synthetase
mog-5	DEAH RNA helicase orthologous to PRP22 proteins.
hrs-1	histidyl-tRNA synthetases (HisRS)
F54D5.11	TFIIE β subunit
xrn-2	5'->3' exonuclease
C48E7.2	RNAPol IIIC homolog
D2085.3	EIF2B epsilon subunit
F55F8.3	WD40-repeat-containing subunit of the 18S rRNA processing complex
Protein degradation	
C33F10.8	F-box protein
pas-4	proteasome α-type seven subunit of the core 20S proteasome subcomplex
Calcium signaling	
mca-3	plasma membrane Ca2+ ATPases (PMCAs)
cal-2	calmodulin homolog
Adhesion/focal adhesion complex	
pxl-1	paxillin 1
pat-2	α-integrin subunit
pat-3	β-integrin subunit
pat-4	integrin-linked kinase
pat-6	α-parvin (Actopaxin)

unc-112	orthologous to human Mitogen inducible gene-2
unc-97	LIM domain-containing protein of the PINCH family
emb-9	α1 chain of Type IV basement membrane collagen
Other adhesion molecules	
hmr-1	classical cadherin
lam-2	laminin gamma subunit
Cytoskeleton	
ifa-3	essential intermediate filament protein
ifb-1	essential intermediate filament protein
tba-1	α tubulin
pfd-3	putative prefoldin, orthologous to human VBP1 that is required for α-tubulin synthesis
tag-170	thioredoxin domain-containing protein orthologous to human TXNDC9
cdk-1	cyclin-dependent kinase, orthologous to CDC28 from S. cerevisiae
fzy-1	an ortholog of S. cerevisiae Cdc20, predicted to regulate metaphase-anaphase transition
knl-1	novel acidic protein, kinetochore component
ani-2	anillins
myo-3	Myosin heavy chain A
Endo/exocytosis	
tom-1	tomosyn ortholog, binds SNAP25 (RIC-4)
unc-11	clathrin-adaptor protein AP180
Mitochondria	
T20H4.5	23 kDa subunit of mitochondrial complex I
Y37D8A.18	mitochondrial ribosomal protein, small

phb-2	mitochondrial prohibitin complex subunit
F43E2.7	mitochondrial carrier Homolog
Signaling pathways	
wrm-1	β-catenin-like proteins
ptc-3	ortholog of Drosophila PATCHED (PTC) and human PTCH
goa-1	heterotrimeric G protein α subunit Go (Go/Gi class)
kin-18	TAO kinase
let-502	Rho-binding Ser/Thr kinase orthologous to human myotonic dystrophy kinase (DM-kinase)
let-92	catalytic subunit of protein phosphatase 2A (PP2A)
Others	
nsf-1	NSF (N-ethylmaleimide sensitive secretion factor) homolog
gfi-2	GEI-4 (Four) Interacting protein
glf-1	UDP-galactopyranose mutase
K12H4.4	signal peptidase complex subunit
F19F10.9	human SART1 homolog
F43G9.10	MicroFibrillar-Associated Protein homolog
pnk-1	PaNtothenate Kinase
C47G2.5	SAPS (phosphatase associated) domain protein
vha-5	subunit a of the membrane-bound (V0) domain of vacuolar proton-translocating ATPase (V-ATPase)
stip-1	STIP (Septin and Tuftelin Interacting Protein) homolog
R03E1.2	KOG ATPase membrane sector associated protein
crn-1	cell death-related 5'-3' exonuclease, homologous to mammalian flap endonuclease 1 (FEN1)

F59E12.11	
T19B10.2	

Table 1. Identified pleiotropic genes that affect touch sensitivity. Gene names and their inferred homologies are listed. The genes were grouped according to their putative functions and/or the functions of their orthologs.

Focal adhesion genes are required for optimal touch sensitivity in the TRNs

Seven of the identified genes (*pat-2*, *pat-3*, *pat-4*, *pat-6*, *unc-97*, *unc-112* and *pxl-1*) encode proteins that comprise the focal adhesions, an integrin-based adhesion complex, suggesting that the focal adhesion genes may be important for mechanosensation. All of these genes cause lethality when mutated due to pharyngeal muscle failure or body-wall muscle failure (Moerman and Williams, 2006; Warner et al., 2011). In the body-wall muscle, these focal adhesion proteins and additional downstream proteins form dense bodies that anchor the myosin filaments to the plasma membrane. We tested additional genes involved in the dense bodies for touch insensitivity by feeding RNAi in TU3595, which expressed SID-1 in all neurons (Figure 2a), and found that Y71G12B.11, which encodes a talin homolog, was also required for optimal touch sensitivity. In contrast, knocking down specialized dense body genes that were not homologous to mammalian focal adhesion components but crucial for body-wall muscle maturation, including *unc-95* and *unc-98* (Broday et al., 2004; Miller et al., 2006), did not affect touch sensitivity (Figure 2a). A non-dense body gene essential for muscle functions (*pat-10*, which encodes troponin C; Terami et al., 1999) and a second α–integrin not involved in dense bodies (*ina-1*) did not affect touch sensitivity either. These results suggest that only the core focal adhesion proteins affect touch sensitivity.

Many of these focal adhesion genes, including *pat-3*, *pat-4*, *pat-6* and *unc-97*, are expressed in the TRNs (Gettner et al., 1995; Hobert et al., 1999; Mackinnon et al., 2002; Lin et al., 2003), suggesting that the focal adhesion genes may function in the TRNs. Indeed, TRN-specific feeding RNAi against *unc-112*, *pat-2* and *pat-3* all produced touch

insensitivity (Figure 2b). Therefore, the focal adhesion genes are needed in the TRNs for optimal touch sensitivity.

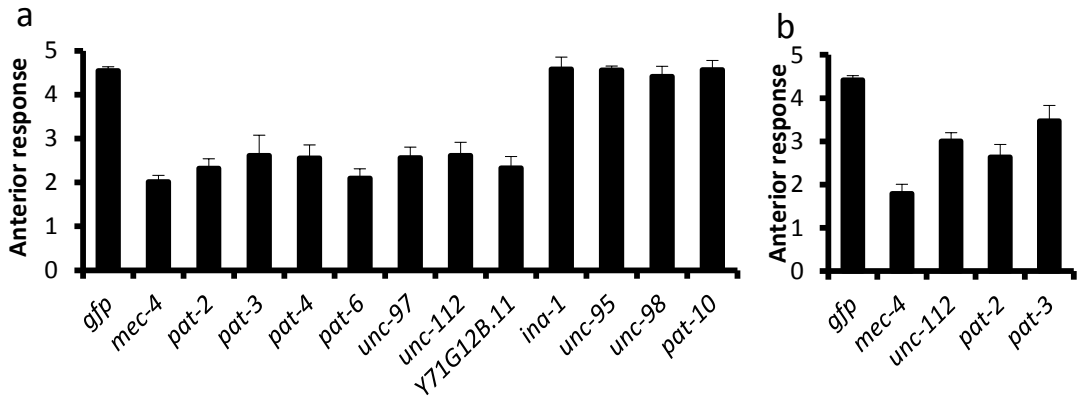

Figure 2. Anterior touch response of animals treated with RNAi against dense body and muscle genes. (a) Anterior touch response (mean ± SEM of the average responses of individual plates tested) of TU3595 fed with RNAi against the indicated genes. At least three independent plates were tested for each gene. Compared to *gfp* RNAi control, the anterior sensitivity of animals treated with RNAi against *mec-4*, *pat-2*, *pat-3*, *pat-4*, *pat-6*, *unc-97*, *unc-112* and *Y71G12B.11* were significantly lower ($p<0.0001$, $N\geq6$). (b) Anterior touch response (mean ± SEM of the average responses of individual plates tested) of TU3568 fed with RNAi against the indicated genes. At least six independent plates were tested for each gene. $p<0.0005$ comparing *mec-4*, *unc-112* and *pat-2* to *gfp*, and $p<0.03$ comparing *pat-3* to *gfp*.

Additional pleiotropic genes needed for optimal touch sensitivity

To confirm additional genes identified in the screen, we tested the touch sensitivity of animals carrying loss-of-function alleles for 12 of the 49 genes, and six of them (*tom-1*, *cdk-1*, *tag-170*, *wrm-1*, *ifb-1*, and *mca-3*; Figure 3) produced touch insensitivity. Although the other six genes did not reduce touch sensitivity, we cannot conclude that they are false positives because the lack of observed touch insensitivity

may be due to the fact that some of these mutations only partially reduce, but do not completely eliminate gene functions. Additionally, the RNAi phenotype could have been caused by secondary targets that were also affected. Indeed, *mec-12* is a known secondary target for the RNAi construct of *tba-1*, so the touch insensitivity caused by RNAi against *tba-1* is likely caused by the reduction of *mec-12*. I have focused on two genes, *tag-170* and *mca-3*, for further analyses.

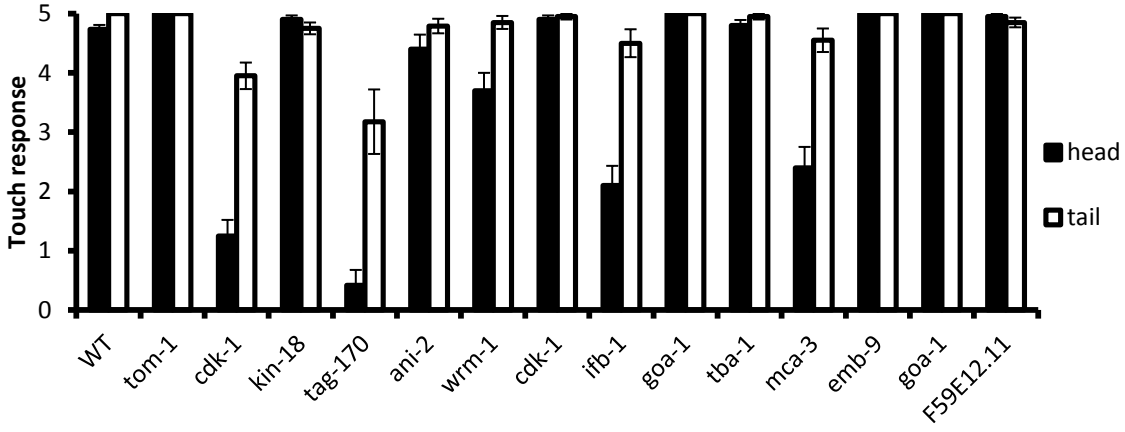

Figure 3. Touch sensitivity of candidate pleiotropic genes that affect touch sensitivity. The anterior (black) and posterior (white) responses to five touches (mean±SEM) are presented. 20 animals were tested for each strain. P<0.0005 comparing the anterior response of *tom-1*, *cdk-1*, *tag-170*, *wrm-1*, *ifb-1* or *mca-3* to *kin-18*.

TAG-170 is required for microtubules in the ALM neurons.

tag-170 encodes a conserved protein involved in microtubule growth and organization (Ogawa et al., 2004; Srayko et al., 2005). Animals carrying the deletion mutation *tag-170 (ok776)* were touch insensitive anteriorly, but retained partial posterior touch sensitivity, suggesting that TAG-170 activity is needed differentially in the TRNs. Wild-type copies of *tag-170* restored the anterior touch sensitivity in *tag-170* animals (Figure 4a), confirming that TAG-170 is needed for touch sensitivity. Consistent with

the role of TAG-170 in microtubule growth, we found that animals homozygous for *tag-170* had reduced levels of MEC-7 β-tubulin and acetylated tubulins in the ALM processes (Figure 4b). TRN-specific feeding RNAi also produced similar reduction in acetylated tubulins, confirming that TAG-170 is required for microtubule assembly. The ALM processes in these animals also lack MEC-2 and MEC-18 usually seen in wild-type processes, although the cell bodies have normal levels of MEC-2 and MEC-18. These defects in MEC-2 and MEC-18 distribution could be caused by the lack of microtubules in these cells (Bounoutas et al., 2009a; Bounoutas et al., 2011). However, the AVM cells in *tag-170* animals have wild-type levels of MEC-2, MEC-18 and MEC-7 in the process (Figure 4b), suggesting that the TAG-170 is needed differentially in the ALM and AVM cells for microtubule assembly.

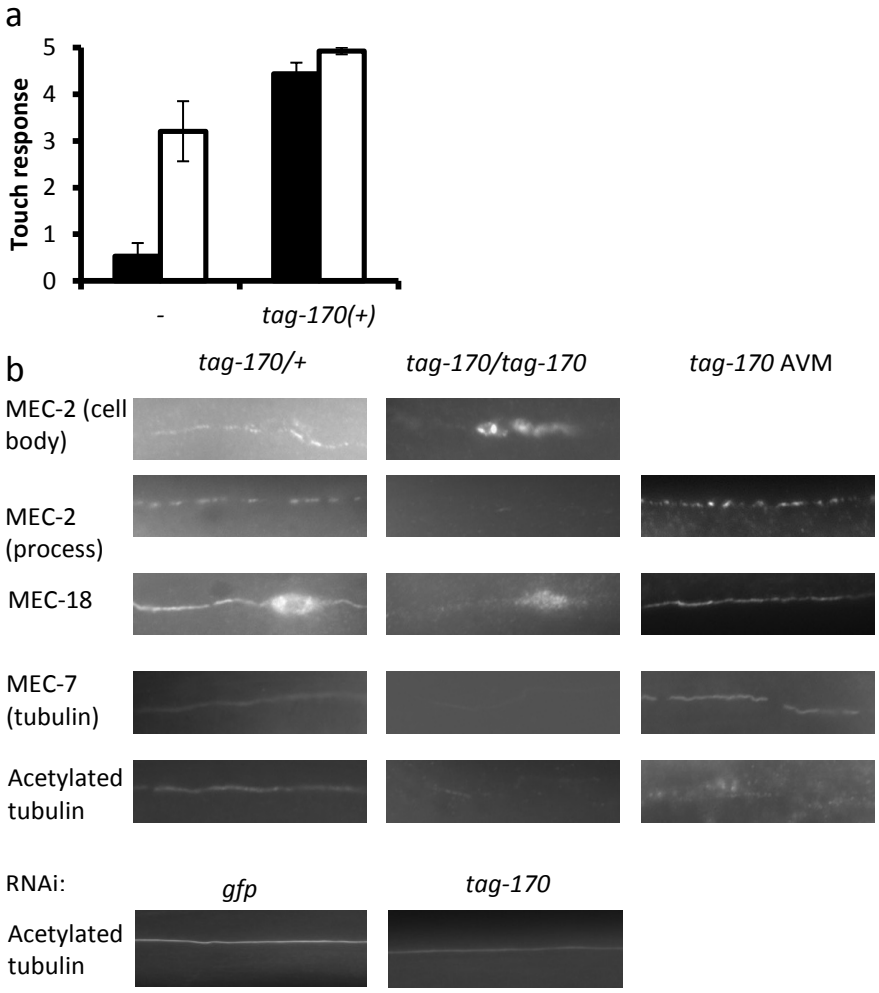

Figure 4. TAG-170 is needed for TRN functions. (a) Anterior (black) and posterior (white) responses to five touches (mean±SEM) of *tag-170(ok776)* animals with (*tag-170(+)*) or without (-) wild-type copies of *tag-170* driven by its own promoter. N>15, $p<0.0001$ for anterior response. (b) MEC-2, MEC-18, MEC-7 and acetylated tubulin staining in ALM cells in *tag-170/+*, *tag-170/tag-170* animals, and wild-type animals treated with TRN-specific feeding RNAi against *gfp* or *tag-170*, and in AVM cells in *tag-170/tag-170* animals.

MCA-3 is needed for touch-induced calcium response in the TRNs

mca-3 encodes a plasma membrane Ca^{2+} ATPase that maintains intracellular Ca^{2+} level by extruding cytosolic Ca^{2+} (Bednarek et al., 2007). *mca-3* is expressed in the

TRNs, as shown by promoter GFP fusion (Figure 5a). Partial loss-of-function alleles of *mca-3* greatly reduced the saturated calcium response elicited by touch (Figure 5b). These cells, however, showed almost wild-type calcium responses to depolarization caused by high potassium in cell culture (data not shown), suggesting that the diminished touch-induced calcium response was caused by defects upstream of calcium signaling, possibly in mechanotransduction.

MCA-3 is required for maintaining low cytosolic Ca^{2+} in the coelomocytes, which is required for their endocytosis functions (Bednarek et al., 2007). Defects in the endocytosis of coelomocytes can be visualized by an increase in GFP signal in the body cavity in a strain expressing secreted GFP (*myo-3p::ssgfp*; Bednarek et al., 2007; Figure 5e). The ALM neurons in *mca-3* mutant animals, however, had wild-type levels of baseline Ca^{2+} levels (Figure 5c). In addition, reducing calcium concentration by growing the animals in the presence of EGTA and without Ca^{2+} did not rescue its anterior touch sensitivity (Figure 5d), although it did restore the endocytosis in coelomocytes, as detected by the absence of secreted GFP in the body cavity (Figure 5e). Therefore the anterior touch insensitivity in *mca-3* mutant animals is unlikely to be caused solely by elevated Ca^{2+} baselines.

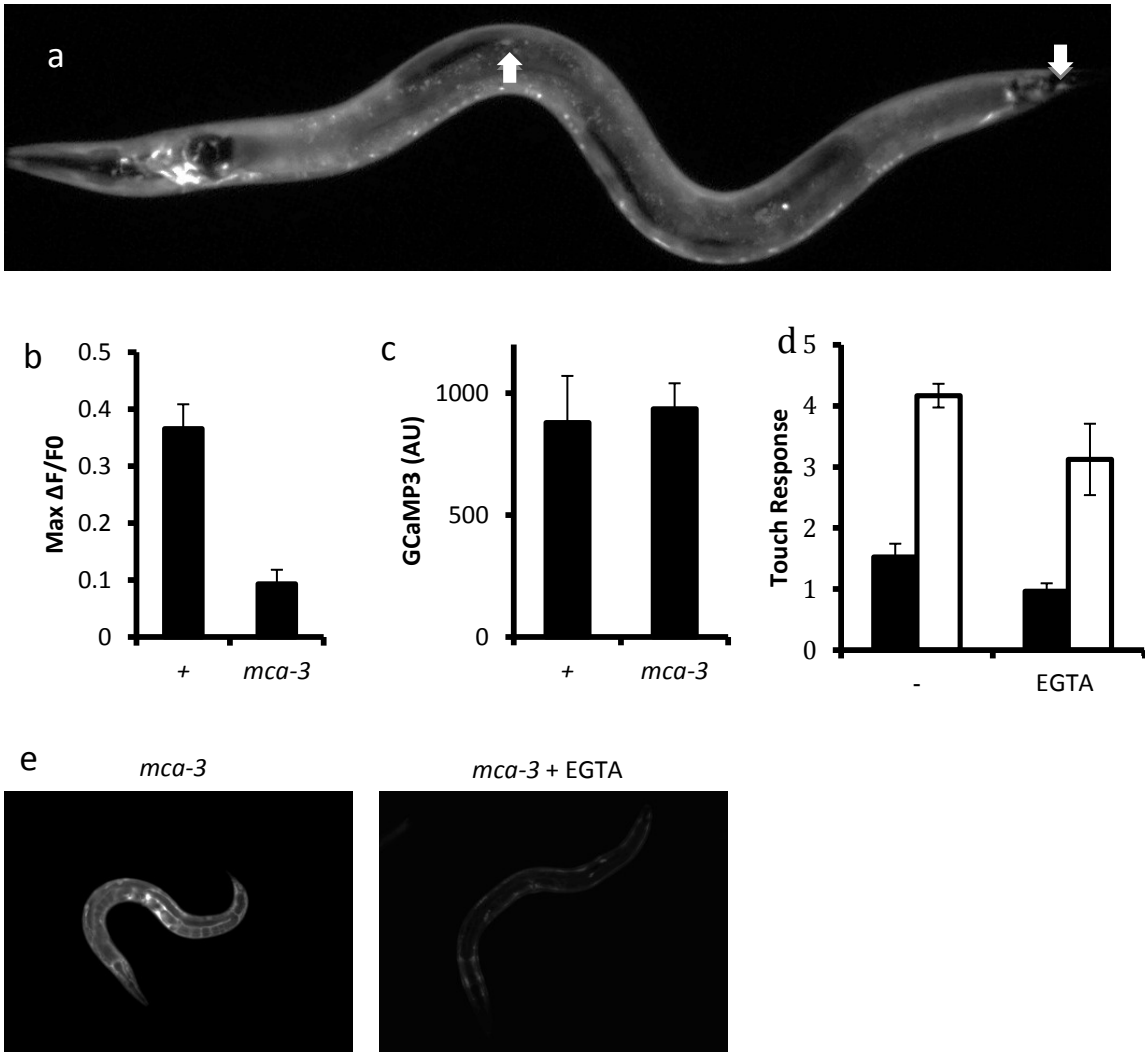

Figure 5. MCA-3 is required for touch sensitivity. (a) *mca-3p::gfp* is expressed in the TRNs. The white arrows mark the ALM and PLM cells. (b) Maximum calcium response (mean ± SEM) elicited by touch in wild-type and *mca-3* animals, as detected by changes of GCaMP3 fluoresence. N≥7. (c) GCaMP3 intensities (mean ± SEM) in ALM neurons in wild-type and *mca-3* animals. N≥5. (d) Anterior (black) and posterior (white) responses (mean±SEM of average responses of independent repeats) to touch in *mca-3* animals with (EGTA) or without (-) EGTA treatment. N≥5. (e) GFP fluorescence in *mca-3; myo-3p::ssgfp* animals with or without EGTA treatment.

Discussion

In this chapter, we have systematically screened pleiotropic genes using tissue specific RNAi, and have identified 61 pleiotropic genes that potentially affect mechanosensation in the TRNs. These 61 genes included the focal adhesion genes, which are needed in the TRNs for optimal touch sensitivity. None of these genes were identified in previous microarray studies to find genes highly expressed specifically in the TRNs (Zhang et al., 2002; Topalidou and Chalfie, 2011). 56 of these genes, however, were shown to be expressed in the TRNs by microarray data (Chaogu and Chalfie, unpublished data). According to the same microarray data, ten genes were expressed higher in the ALM neurons and four were expressed higher in the PLM neurons (5% false detection rate (FDR); Chaogu and Chalfie, unpublished data). This result is expected because we only tested the anterior touch sensitivity. In addition, we further tested 12 genes with viable alleles and confirmed that six of them produced touch insensitivity. Our results suggest that a large number of genes previously missed by saturated forward genetic screens also affect touch sensitivity.

Screening pleiotropic genes using tissue-specific feeding RNAi

To identify pleiotropic genes affecting mechanosensation, we performed tissue-specific feeding RNAi in animals expressing SID-1 only in the neurons. We further tested 12 of the identified genes using available alleles, but only six of them produced touch insensitivity, suggesting that the false positive rate may be as high as 50%. However, of the six mutant alleles that did not produce touch sensitivity: *kin-18(ok395)* and *ani-2(ok1147)* were likely null deletions, but both showed maternal rescue effects and therefore may have had residual activities; *emb-9(hc70)* and *tba-1(or346)* were

temperature-sensitive alleles and therefore not null alleles; Only *goa-1(sa734)* and *F59E12.11(tm3828)* were likely a null, which contained an early stop codon. Therefore, the lack of touch insensitivity in animals carrying these alleles could be caused by residual activities. Furthermore, we have not sequenced the RNAi constructs to confirm that they are indeed correctly labeled, so that some of these RNAi constructs may actually target other genes. If we take these factors into consideration, the real false positive rate may be much lower than our estimation.

If we exclude the 12 housekeeping genes related to transcription and translation, which may affect touch sensitivity by disrupting the general functions of the TRNs, and use a conservatively estimated false positive rate of 50% (six out of 12 genes), still 2.5% of the genes screened produced touch insensitivity. This ratio is much higher than genes needed for touch sensitivity as identified by forward genetic screens (18/19000=~0.1%), suggesting the importance of pleiotropic genes in mechanosensation. The genes discovered in this type of screen, however, are not guaranteed to cause touch insensitivity by affecting mechanotransduction. The pleiotropic effects associated with mutating these genes suggest that they may affect general cellular functions, and mutations in these genes could have reduced touch sensitivity because the general health or functionalities of the TRNs or downstream neurons were compromised. In our screen, we have identified possibly 15 such genes, 12 likely needed for general transcription/translation, and at least an additional three genes (T20H4.5, Y37D8A.18 and F43E2.7) likely needed for basic mitochondrial functions. Reductions in the activities of these genes likely caused general cellular dysfunctions in the TRNs and may be irrelevant to mechanotransductions.

The higher discovery rate in our screen compared to forward genetic screens could also partially be caused by increased sensitivity associated with the method. Because each gene is knocked down and tested in multiple independent plates, each with multiple animals, we were able to detect subtler phenotypes than was possible with a mutagenesis screen, in which at most 25% of the animals on a plate would show a phenotype if the F1s were singled out following mutagenesis. The extra sensitivity, however, may also increase false positive rate and the detection rate of irrelevant genes that affected touch response by interfering with downstream neuronal functions. Therefore, tissue-specific RNAi screen is useful at initially detecting pleiotropic genes affecting mechanosensation. Associating these genes with the mechanosensation requires detailed analysis and confirmation of each candidate target.

Pleiotropic genes affecting TRN mechanosensation

Most of the 61 genes we have identified can be categorized into eight groups. In addition to genes affecting transcription/translation and mitochondria functions, which may affect general TRN functions, the other genes may be involved in protein degradation, calcium signaling, adhesion complex, cytoskeleton, endo/exocytosis, and classical signaling pathways. Further testing of mutant alleles and previous reports confirmed genes in at least four of these six groups: calcium signaling (*mca-3*; Figure 3), adhesion complex (*unc-97*; Hobert et al., 1999), cytoskeleton (*tag-170* and *cdk-1*; Figure 3) and endo/exocytosis (*tom-1* and *unc-11*; Figure 3; M. Chalfie, unpublished data).

Our result that *mca-3* was needed for touch sensitivity suggests that calcium signaling modulates touch sensitivity. Kindt et al. (2007) have reported similar findings with regard to the effect of calcium on ALM habituation. PMCA2, A rat ortholog of

mca-3, extrudes Ca^{2+} from the stereocilia of both inner and outer hair cells (Crouch and Schulte, 1995; Yamoah et al., 1998). Mutations in Pmca2 produce deafness and balance defects in rats (Street et al., 1998; Bortolozzi et al., 2010). These reports suggested that PMCAs may play a conserved role in mechanosensation across species.

The finding that cytoskeleton genes affect touch sensitivity is unsurprising, because the TRNs contain specialized microtubules essential for mechanosensation. Indeed, our data indicated that TAG-170 likely affects touch sensitivity by organizing microtubules in the ALM neurons. In support of this hypothesis, microarray data indicate that *tag-170* is expressed at a higher level in the ALM neurons than in the PLM neurons (1.8 fold change, 2% FDR; Zheng and Chalfie, unpublished data). In addition, we have identified three cell-cycle related genes (*cdk-1*, *fzy-1* and *knl-1*). Although mutations in these genes could potentially affect the development of the TRNs by interfering with cell division, this hypothesis is unlikely to occur in our RNAi screen since the RNAi effect took place after almost all cells in the touch circuit had arisen (with the exception of the AVM neurons, which are dispensable for touch sensitivity until late in development; Chalfie et al., 1985). We hypothesize that the products of these genes may instead organize the specialized microtubules in the TRNs.

In addition to microtubule-related cytoskeleton components, we have also identified two intermediate filament genes, *ifa-3* and *ifb-1*, that co-express in vivo and form intermediate filaments in vitro when mixed together (Karabinos et al., 2003). Because of the similarities among intermediate filament genes, the RNAi bacterial strains that targeted these two genes likely targeted other genes encoding intermediate filaments together, and therefore we cannot conclude that *ifa-3* and *ifb-1* are specifically required

for mechanosensation. Nevertheless, our result indicates that the intermediate filaments affect touch sensitivity. Intermediate filament genes are mainly expressed in non-neuronal tissues, such as the hypodermis, where they connect with Fibrous Organelles (FOs) that anchor the body wall muscle and the TRNs to the hypodermis (Karabinos et al., 2001; Karabinos et al., 2002). Therefore it is likely that leaking RNAi effects in the hypodermis disrupted the attachment between the TRNs and the hypodermis through the FOs, which reduced touch sensitivity. In the body wall muscle, the FOs are attached to the integrins expressed in the muscle cells through spectraplakins VAB-10 (Labouesse, 2006). Because RNAi against *pat-2* and *pat-3*, which encode the α- and β-integrins, also caused touch insensitivity, we hypothesize that the integrin-based adhesion complexes in the TRNs may be similarly attached to the FOs, and such attachment is required for touch sensitivity.

Focal adhesion proteins are needed for optimal touch sensitivity

The focal adhesions are integrin-based adhesion complexes. Because knocking down other genes essential for muscle functions, including *unc-95*, *unc-98* and *pat-10* (Terami et al., 1999; Broday et al., 2004; Miller et al., 2006), did not cause touch insensitivity, the touch insensitivity seen in animals with reduced activities of the core focal adhesion genes was not a secondary effect of leaking RNAi in the body wall muscle. This is further supported by TRN-specific feeding RNAi against *pat-2*, *pat-3* and *unc-112* in TU3568, all of which reduced touch sensitivity. These results suggest that the focal adhesion proteins may have specific functions in TRN mechanosensation.

Both UNC-95 and UNC-98 act late in the assembly of adhesion complexes in the body wall muscle, and null alleles of these genes cause less severe phenotypes than the

Pat phenotype seen in *pat-2*, *pat-3*, *pat-4*, *pat-6*, *unc-112* and *unc-97* mutants. UNC-95 maintains the organization of dense bodies and acts upstream of vinculin (Broday et al., 2004). UNC-98 is needed to anchor the thick filaments to the M-lines (Miller et al., 2006). The fact that they do not contribute to touch sensitivity suggests that the focal adhesion proteins in the TRNs form a different complex than the dense bodies in the body wall muscle. This is expected because the dense bodies are huge structures specialized in anchoring actin filaments, with a length larger than the diameter of the TRNs (Figure 6). No such electron-dense structure was observed in the TRNs under electron microscopy. Therefore, the core focal adhesion proteins likely form a different complex in the TRNs.

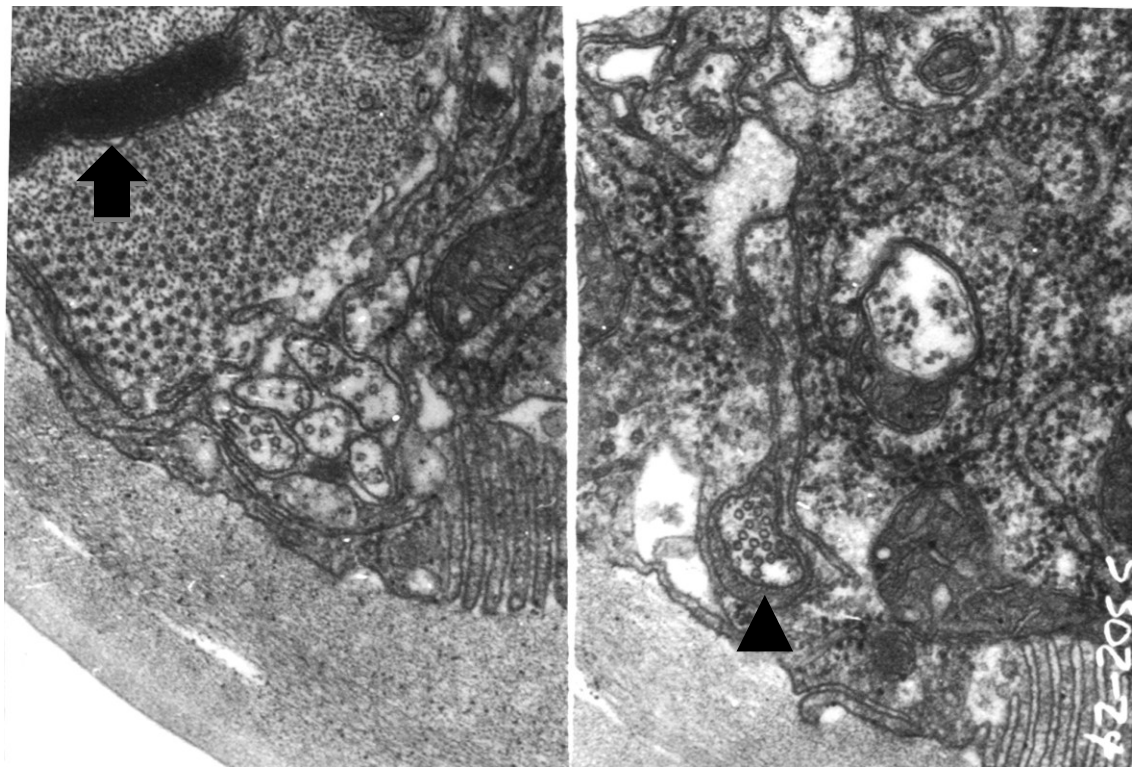

Figure 6. TEM images of a dense body and an ALM process. The dense body and the ALM process in a wild-type adult animal are marked with an arrow and an arrowhead, respectively. (M. Chalfie, unpublished data; See also Chalfie and Thomson, 1979).

In addition to functioning as mechanical anchors, focal adhesions also sense stretching force and activate downstream signaling molecules (Chen, 2008; Vogel and Sheetz, 2009). In the TRNs, the focal adhesion proteins can function either as a direct mechanical linkage in gating mechanotransduction channels, as an indirect mechanical support that strengthens the TRN process, or as a signaling pathway activated by integrin ligands and mechanical force to modulate mechanotransduction through the MEC-4 channel. Because RNAi treatments do not completely eliminate gene functions, we do not know if complete elimination of the focal adhesion proteins completely abolish touch sensitivity, as would be predicted if they acted directly as the gating mechanism. Alternatively, they could act as modulatory structures that fine-tune force sensitivity and/or the gain of mechanotransduction, either mechanically or chemically. Identifying the null phenotypes of these genes would be crucial in determining their roles in mechanosensation.

Experimental procedures

Strains

C. elegans strains were maintained at 15˚C as described by Brenner (1974). For EGTA treatment, animals were grown on NGM plates without Ca^{2+} and supplemented with 1 mM EGTA as described by Bednarek et al. (2007). All strains used are listed in Appendix II.

Feeding RNAi screen

The list of lethal RNAi was pulled from WormMart using the following phenotype codes: lethal, adult_lethal, embryonic_lethal, embryonic_terminal_arrest_variable_emb, embryonic_lethal_late_emb, larval_lethal, larval_arrest, late_larval_lethal, late_larval_arrest, paralyzed, one_cell_arrest_early_emb, and embryonic_terminal_arrest_variable_emb. The search was restricted to RNAi experiments described by Fraser et al. (2000) and Kamath et al. (2003), which used the Ahringer RNAi library.

Feeding RNAi treatments were performed as described by Calixto et al. (2010a) in TU3595 for the screen and in TU3568 for TRN-specific RNAi. Animals were grown on 6-well plates instead of 5 cm plates to save space. Each RNAi bacterial strains was tested five times independently, and genes that produced touch insensitivity in at least four out of five tests were considered as causing touch insensitivity. For a single test, we estimated the false positive rate to be <20% from the first two round of tests. Therefore, the false positive rate of the screen caused by the touch assay was estimated to be

$C_5^1 \times 0.2^4 \times 0.8 + 0.2^5 = 0.67\%$, corresponding to $0.67\% \times 908 = 6$ false positives out of the 61 genes obtained from the screen.

Touch sensitivity assay

We tested touch sensitivity using a method modified based on the one described by Chalfie and Sulston (1981). Instead of touching the animal by sliding a thin hair across the top of the worm, we gently touched the side of the animal. Each animal was tested five times anteriorly and five times posteriorly (Hobert et al., 1999), or five times anteriorly only for RNAi tests, since feeding RNAi is less effective for posterior touch sensitivity (Calixto et al., 2010a). 10 to 20 animals were tested each time. The average and SEM of the response of animals were reported. P-values were calculated using Student's t test.

Calcium imaging

Each animal was glued on its ventral side using Dermabond (Ethicon Inc., Somerville, NJ) onto a 4% agarose pad in M9 buffer on a 24 mm × 60 mm No. 1 coverslip, and covered with 100 µl M9 buffer. We mounted the coverslip on a rotating stage on a Zeiss Observer Z1 microscope equipped with an Eppendorf TransferMan NK2 micromanipulator (Eppendorf North America, Hauppauge, NY). A glass probe driven by a piezo lever was mounted on the micromanipulator, and carefully placed next to the worm without pressing the worm. The piezo lever was driven by 250 ms square waves from a 33221A waveform generator (Agilent Technologies Inc., Santa Clara, CA) through a Piezo Linear Amplifier (Piezo Systems Inc., Woburn, MA) with the gain set to

10X. During the recording, we illuminated the animal with a 470 nm LED from Colibri 2 (Carl Zeiss Microscopy LLC, Thronwood, NY) at 10% intensity through a GFP filter cube, and recorded the images through a Zeiss Apochromat 40× 0.95 air objective with a Photometric Evolve 512 camera at ~10 fps and analyzed with Axiovision (Carl Zeiss Microscopy LLC, Thornwood, NY).

Microscopy

Animals were immobilized on 4% agarose pads with 30 mM sodium azide and imaged on a Zeiss Observer Z1 inverted microscope using a Zeiss Apochromat 40x 0.95 air objective for TRN antibody staining, or a Zeiss Neofluor 10× 0.3 objective for the remaining images. The images were acquired with a Photometrics Coolsnap HQ2 camera using Zeiss Colibri 2 as excitation source.

Chapter III. Long-term sensitization of *C. elegans* mechanosensation through a secondary integrin-based mechanosensory system

Abstract

Modulation of mechanosensation enables animals to distinguish useful signals from background stimuli. We have identified a second mechanosensory system utilizing integrin/focal adhesion signaling that modulates touch sensitivity in the *C. elegans* touch receptor neurons (TRNs), which sense gentle touch along the body through the MEC-4 DEG/ENaC mechanotransduction channels. Integrin signaling increases anterior touch sensitivity by lowering the detection threshold to touch and maintaining normal response to mechanical stimuli after sustained background vibration. Because this modulation occurs independently of MEC-4 channel activity and neuronal activity, the integrins function as secondary force sensors. Integrin signaling acts upstream of AKT-1 and DAF-16 in parallel with insulin signaling, which also modulates touch sensitivity. Each signaling pathway can compensate for defects in the other in modulating touch sensitivity. Our findings demonstrate a new form of long-term modulation that maintains mechanical sensitivity under sustained background vibration, counteracting the effect of habituation.

Introduction

Sensory perception changes so animals can adapt to different environments. For example, previous exposure to odors reduces excitatory responses to odors (Buonviso and Chaput, 2000), and non-visual stimuli such as sound or motor activity enhance visual perception or create visual illusions (Schutz-Bosbach and Prinz, 2007; Shams and Kim, 2010). Modulation of sensory cells can also increase the dynamic range of detected stimuli. In the mammalian visual system, cone and rod cells detect visual signals with intensities varying over ten orders of magnitude through regulation of intracellular calcium levels and bleaching/regeneration of photopigments (Fain et al., 2001). Modulation of sensory transduction optimizes detection of sensory stimuli under various conditions.

Mechanosensation is modulated in multiple ways. Mammalian hair cells and skin touch receptors adapt to sustained activation and reduce channel currents, thus maintaining sensitivity under constant activation (Eatock, 2000; Holt and Corey, 2000; Lumpkin et al., 2010). Both hearing and touch sensation also habituate to repeated stimuli (Wickesberg and Stevens, 1998; Pinsker et al., 1970). In addition to adaptation and habituation, mechanosensation can be sensitized by mechanical stimuli. Long term exposure to moderate-level sound sensitizes hearing and protects hearing from subsequent traumatic exposure in guinea pigs (Kujawa and Liberman, 1999). Similarly, intermittent vibration hypersensitizes touch sensitivity in rats (Govindaraju et al., 2006). However, the mechanism of how mechanical stimuli sensitize mechanosensation remains unknown.

In this paper we show that sensitivity to gentle touch along the body is modulated in *C. elegans*. Gentle touch is sensed by six touch receptor neurons (TRNs), and genetic screens have identified several genes needed for mechanosensation (Chalfie and Sulston, 1981; Chalfie and Au, 1989). Some of these genes encode components of the MEC-4 DEG/ENaC channel complex that transduces touch (O'Hagan et al., 2005). The TRNs sense changes in applied force and adapt quickly to constant pressure (O'Hagan et al., 2005). The touch response also adapts to repeated stimuli over the course of several minutes and sensitizes for a short time (~2 min) following a single strong stimulus (Rankin et al., 1990). These short-term changes in TRN activity likely reflect changes in calcium handling subsequent to the touch (Suzuki et al., 2003), not changes in force sensitivity. We report here that TRN touch sensitivity can be modulated on a longer time scale (hours rather than minutes) either negatively by prolonged exposure to high-salt conditions or positively by vibration. The effect of vibration requires integrin signaling.

Integrins and additional proteins form focal adhesions, mechanosensory complexes that transmit and sense forces between a cell and the extracellular matrix (Chen, 2008). Integrins sense cellular stretching forces, reinforcing adhesion (Roca-Cusachs et al., 2009) and inducing long-term cellular changes (Vogel and Sheetz, 2009). The role of integrins and other focal adhesion proteins in neuronal mechanosensation, however, is unclear. Focal adhesion proteins are expressed in mechanosensory cells, including the TRNs and the vertebrate hair cells (Gettner et al., 1995; Hobert et al., 1999; Littlewood Evans and Muller, 2000; Mackinnon et al., 2002; Lin et al., 2003), but they are not likely the primary sensors in mechanosensory neurons because integrin-mediated mechanosensation is usually slow (tens of seconds;Vogel and Sheetz, 2009), whereas

mechanosensory neurons respond rapidly to mechanical signals (<1 msec in *C. elegans* TRNs, vertebrate hair cells, and fly bristles, and 0.5 - 1.5 msec in Pacinian corpuscles; Gray and Malcolm, 1950; Corey and Hudspeth, 1979; Walker et al., 2000; O'Hagan et al., 2005).

Here we show that integrin signaling modulates the force sensitivity and consequent calcium response to touch in the anterior TRNs, the ALM cells, and is needed for the sensitization caused by vibration. Such sensitization maintains sensitivity under background vibration. Because this integrin-dependent sensitization occurs without functional MEC-4/MEC-10 mechanotransduction channels, focal adhesions serve as direct or indirect components of independent, secondary mechanosensors.

Results

High salt and vibration modulate touch sensitivity

Wild-type animals grown with excessive salt (230 mM NaCl vs 50 mM under normal conditions) had reduced anterior touch sensitivity (Figure 7a). The reduced anterior touch sensitivity, however, can be restored by subjecting animals to sustained vibration (50 Hz with peak acceleration of 1.5 g; see Figure 8 for vibration parameters) for at least two hours (Figure 7a/8a). Sustained vibration also increased anterior touch sensitivity of wild-type animals grown under normal conditions, as detected by a nearly two-fold increase in sensitivity to force measured by calcium imaging: the probe displacement required for 50% activation (D_{50}), which is proportional to the force applied, decreased to 0.61±0.18 μm (N=7, $p<0.05$) in animals raised with sustained vibration for two hours from 1.11±0.12 μm (N=11) in animals that did not undergo this treatment (Figure 7b). The smallest displacement needed to elicit a calcium response (defined as 10% relative activation above the baseline) in the ALM neurons was also smaller in animals under sustained vibration ($D_{control}$=0.43 ± 0.06 μm vs D_{vib}=0.25 ± 0.03 μm, p=0.01; Figure 7c). In addition, ALM neurons of treated animals produced spontaneous calcium spikes without a mechanical stimulus; no such spikes were observed in untreated animals (Figure 7c). The appearance of the spikes suggests that the vibrated animals detected either background vibrations from the microscope stage or stimuli induced by the contraction of body wall muscles. Thus, prolonged vibration lowers the force detection threshold of ALM neurons.

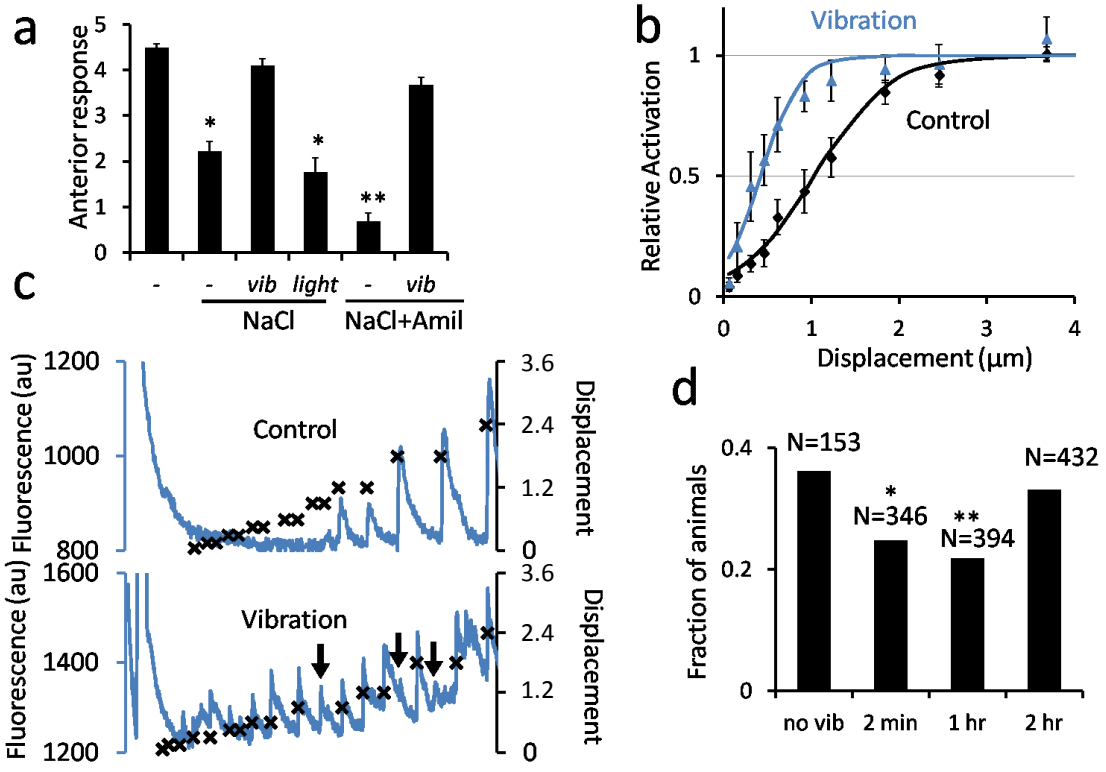

Figure 7. Touch sensitivity of wild-type animals after sustained vibration. (a) Anterior touch sensitivity (mean ± SEM) of wild-type animals under normal (-), sustained vibration (vib) or sustained light activation (light). Additional treatment is noted below: 230 mM NaCl (NaCl) or 230 mM NaCl with 2 mM Amiloride during vibration and rested for 1 hr on 230 mM NaCl without Amiloride before testing (NaCl+Amil). *p<0.0001 compared to wild-type response. **p<0.0001 compared to vib under NaCl+Amil. (b) Normalized calcium responses of wild-type animals with (blue) or without (black) vibration under different probe displacements, and their corresponding Boltzmann fits. N≥7 for all strains. Error bars represent SEM of responses at each given force. (c) Sample calcium response raw traces (blue) from wild-type animals with (down) or without (up) vibration. The displacement of each stimulus (black cross) is marked at each peak. Arrows indicate calcium peaks without stimulus. (d) Fractions of animals moving backward in response to pulse vibration without sustained vibration (no vib), or with sustained vibration for 2 min - 0.5 hr (2 min), 1 - 1.5 hrs (1 hr) or 2 - 2.5 hrs (2 hr). Total number of animals tested were noted on top of each bar. *p<0.015, **p<0.0001 compared to “no vib” using Fisher’s exact test.

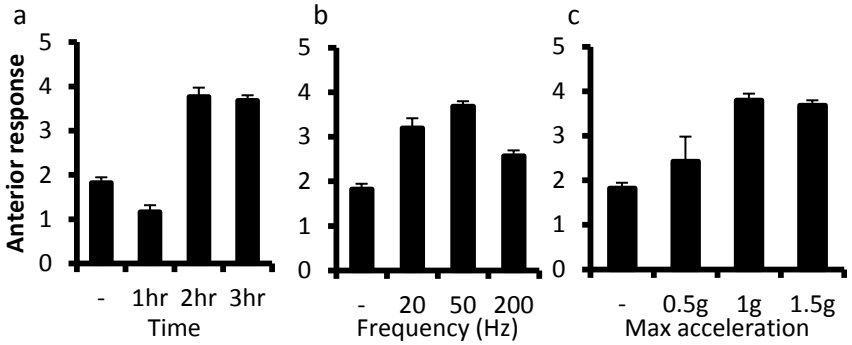

Figure 8. Parameters for vibration-enhanced touch sensitivity. The touch-sensitivity enhancement is most significant when the animals are vibrated at 50Hz, >1g max acceleration for more than two hours, as shown in the following panels: (a) Anterior sensitivity (mean ± SEM) of wild-type animal with or without (-) vibration at 50Hz, 1.5g max acceleration for the indicated time. (b) Anterior sensitivity (mean ± SEM) of wild-type animal with or without (-) vibration at 1.5g max acceleration for 3 – 12 hours at the indicated frequency. The results for vibration for three hours and 12 hours were the same and were pooled together. (c) Anterior sensitivity (mean ± SEM) of wild-type animal with or without (-) vibration for three hours at 50Hz and the indicated max accelerations. For all panels, *$p<0.0001$, **$p<0.0005$, ***$p<0.001$.

Acute, low frequency stimulation (for example, 1.5 s buzz stimulations at 60 s intervals) of the TRNs leads to habituation (Suzuki et al., 2003), yet prolonged vibration enhanced sensitivity. These two counteracting effects led us to hypothesize that vibration-enhanced touch sensitivity may compensate for the loss of touch sensitivity caused by background vibration, therefore maintaining anterior touch sensitivity under diverse conditions. To test this hypothesis, we measured the touch sensitivity of wild-type animals in the presence of sustained vibration. We were unable to test touch sensitivity directly on vibrating plates, because touching the agar surface close to an animal with an eyebrow hair on a vibrating plate induced strong backing behaviors. To circumvent this problem, we stimulated animals with a strong half-second pulse during

the sustained weaker vibration (see Experimental Procedures). Thirty six percent of wild-type animals (N=153) responded to the pulse by backing when no sustained vibration was present (Figure 7d). Fewer animals (22%, N=394) responded by backing when the pulse was given after exposure to a weaker sustained vibration for a period of less than two hours. After two hours of sustained vibration, however, animals responded similarly to animals that had not seen sustained vibration (33%, N=432), possibly because they became sensitized. These results suggest that the enhanced touch sensitivity restores touch sensitivity under sustained background vibration to normal level.

The vibration-induced sensitization did not require the MEC-4 mechanotransduction channels or electrical activation of the TRNs, since vibration enhanced touch sensitivity even when the MEC-4 channels were blocked with amiloride (Figure 7a), and activation by channelrhodopsin did not enhance touch sensitivity (Figure 7a). These data suggest that vibration is sensed through a different force sensor. Since reduction of focal adhesion proteins by RNAi or partial loss-of-function mutations causes touch insensitivity (Hobert et al., 1999; Calixto et al., 2010a) and many focal adhesion proteins are expressed in the TRNs (Figure 11a), we investigated, as described in the following sections, whether the focal adhesion proteins were the secondary force sensor needed for vibration-enhanced sensitivity.

Focal adhesion proteins modulate TRN touch sensitivity

Because RNAi often reduces but does not eliminate gene expression, our previous experiments (Calixto et al., 2010a) indicated that focal adhesion proteins were needed for touch sensitivity, but not whether they were essential for it. Most focal adhesion genes are expressed in the TRNs (Gettner et al., 1995; Mackinnon et al., 2002; Lin et al., 2003;

Hobert et al., 1999; Figure 11a), but mutations in these genes cause lethality because of their functions in the body-wall muscles. To circumvent the embryonic lethality associated with the complete loss of expression of the focal adhesion genes, we tested touch sensitivity in animals that were mosaic for null alleles (Figure 9a). Loss of *pat-2, pat-3, unc-97, unc-112,* and *pat-6* in the anterior TRNs (the two ALM cells and the AVM cell) yielded animals that were substantially insensitive to anterior touch. The loss of these genes in the anterior TRNs, however, did not produce the complete touch insensitivity seen in mosaics with a *mec-4(u253)* mutation. The residual touch sensitivity was not likely due to perdurance, since the first common precursor is five generations previous for the ALM cells and ten generations previous for the PLM cells. These data suggest that the focal adhesion proteins are not essential for mechanotransduction, but are important for optimal touch sensitivity.

Loss of the integrins and other focal adhesion proteins in the TRNs did not result in general cellular dysfunction. ALM neurons expressing channelrhodopsin-2 (Nagel et al., 2005) and containing or lacking the rescuing arrays of the focal adhesion genes were equally capable of inducing backing when activated by blue light (Figure 9b). In contrast, animals with reduced activity of the L-type voltage-gated calcium channel EGL-19 responded less to blue light activation of channelrhodopsin-2. These data indicate that the focal adhesion proteins do not disrupt the channelrhodopsin-2 response and are likely to act early in mechanosensation.

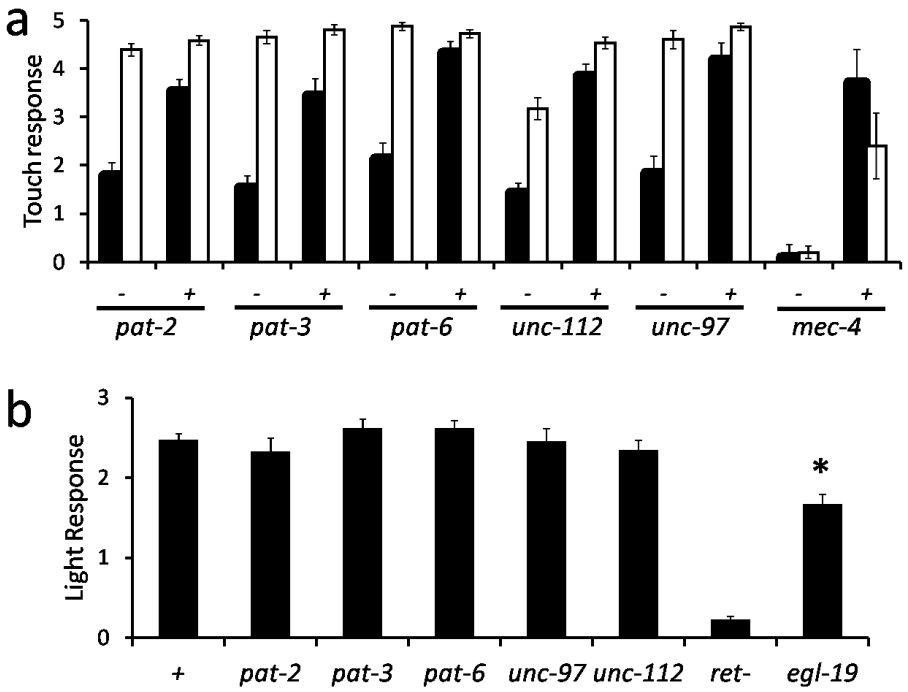

Figure 9. TRN sensitivity of focal adhesion mutants. (a) Anterior (black) and posterior (white) responses (mean ± SEM of responses of individual animals) of mosaic *pat-2(ok2148), pat-3(st564), pat-6(st561), unc-112(gk1), unc-97(ra115)* and *mec-4(u253)* animals with (+) or without (-) the rescuing arrays in the TRNs. For the focal adhesion genes, N>20 for anterior responses, and N>15 for posterior responses. For *mec-4*, N>10. For all anterior responses, $p<0.0001$ between (+) and (-) animals. (b) Response (mean ± SEM of responses of individual animals) to three light pulses from focal adhesion mosaic animals lacking the rescuing arrays in the TRNs but expressing channelrhodopsin in the TRNs and from *egl-19* animals expressing channelrhodopsin in the TRNs. N≥20 for all strains tested. Compared to the wild type, *$p<0.001$.

The TRNs lacking rescuing arrays had normal morphology except for minor migration defects seen in ALM cells lacking *pat-3* and loss of attachment to the hypodermis in all focal adhesion mutants (Figure 10a/b; Figure 11b; Gettner et al., 1995). The loss of attachment, however, cannot solely account for the reduced touch sensitivity; ALM processes with reduced *unc-112* or *pat-6* activity (through neuronally-enhanced

RNAi) displayed normal attachment (Figure 11c), yet the mutant animals were less sensitive to touch than controls. The migration defect seen in *pat-3* animals could not account for the reduced touch sensitivity either, because animals lacking the second *C. elegans* α-integrin gene, *ina-1*, were touch sensitive despite having similar migration defects (Baum and Garriga, 1997; Figure 11d/e).

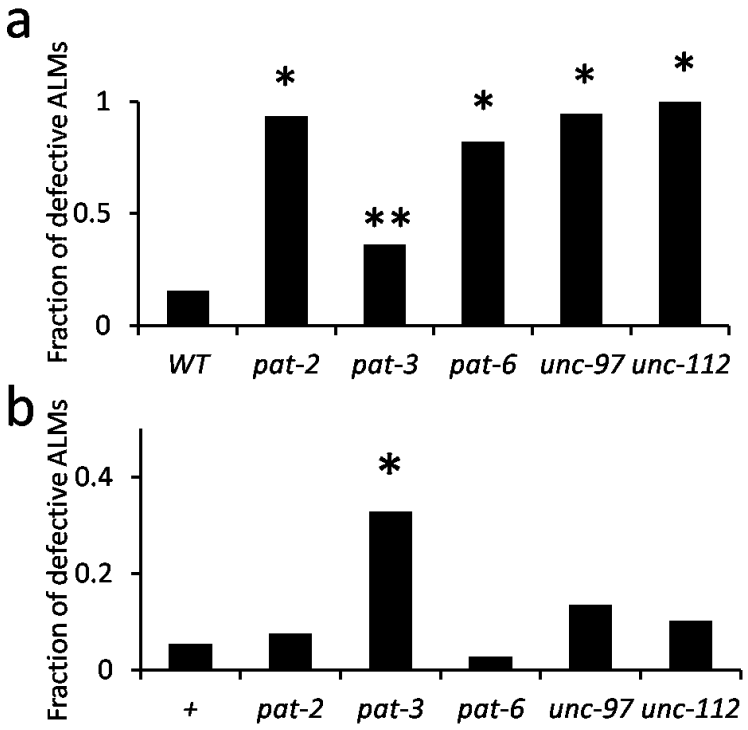

Figure 10. TRN developmental defects in focal adhesion mutants. (a) Fraction of TRNs showing attachment defects. N>15. *p<0.0001, **p=0.03, calculated using Fisher's exact test. (b) Fractions of ALM neurons showing migration defects in wild-type and mosaic animals. N≥20 for all animals. *p<0.001 between *pat-3* mosaic animals and wild-type animals using Fisher's exact test. See Experimental Procedures for scoring standards.

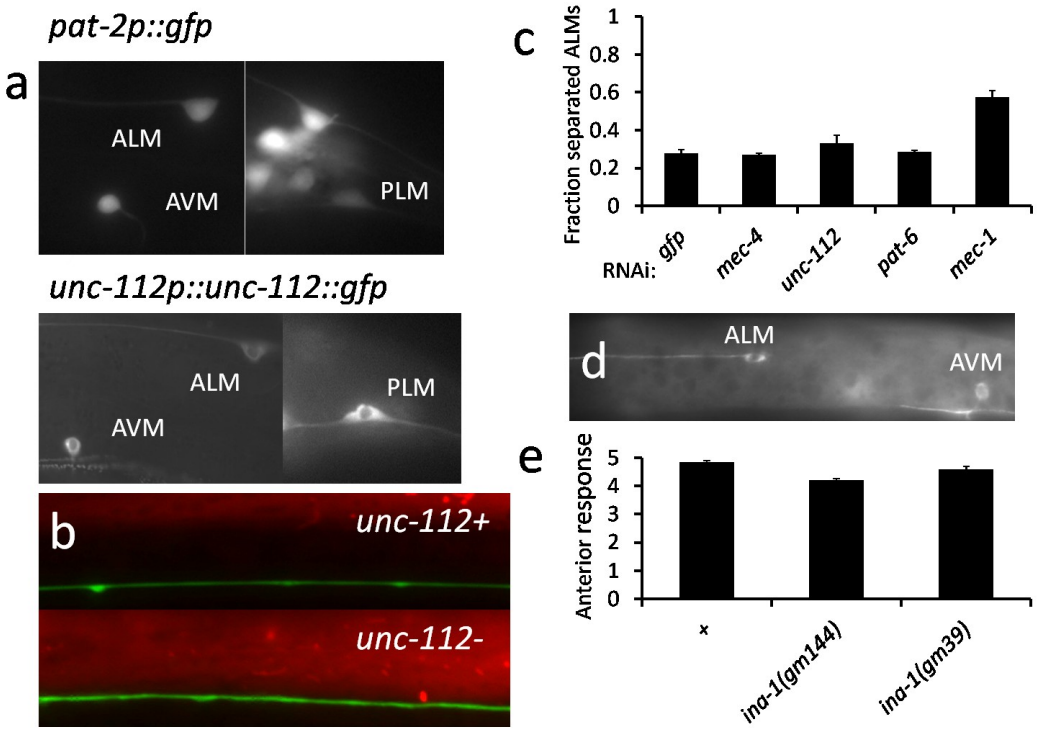

Figure 11. The expression of focal adhesion genes in the TRNs and developmental defects of ALM neurons in integrin and focal adhesion mutants. (a) The expression of *pat-2p::gfp* or *unc-112p::unc-112::gfp* with RNAi against *gfp* is shown in the pictures. Because of the inefficient systemic RNAi in the nervous system, only *gfp* expression in the body-wall muscle, but not in the neurons is reduced. ALM, AVM and PLM cells are labeled in the pictures. (b) ALM processes (green) and the body wall muscle (red) in *unc-112* mosaic animals with or without *unc-112* in the ALM cells. The ALM process is normally attached to the hypodermis, which would separate it from the body-wall muscle at the L4 stage. ALM processes without attachment to the hypodermis would appear adjacent to the body-wall muscle. The ALM cell body would also be pressed against the body-wall muscle, assuming a half-circle shape instead of the normal raindrop shape. (c) Fractions of detached ALM cells in animals treated with RNAi against *gfp, unc-112, pat-6* or *mec*-1. See Experimental Procedures for detailed scoring standard. $p<0.005$ between *mec-1* RNAi and any of the other RNAi treatment. (d) an ALM cell with migration defect in *ina-1(gm144)* animals. e) Anterior sensitivity (mean ± SEM) of wild-type, *ina-1(gm39)* and *ina-1(gm144)* animals.

Focal adhesion proteins did, however, modulate the force sensitivity of the ALM neurons as measured by a decrease in touch-stimulated uptake of calcium (Figure 12a). The displacement needed to achieve 50% activation (D_{50}) of the TRNs was higher in *pat-2* mutants (2.64±0.18 µm, mean ± SEM, N=11, p<0.0001) and *unc-112* mutants (1.72±0.18 µm, N=6, p=0.01) than wild-type animals (1.11±0.12 µm, N=11).

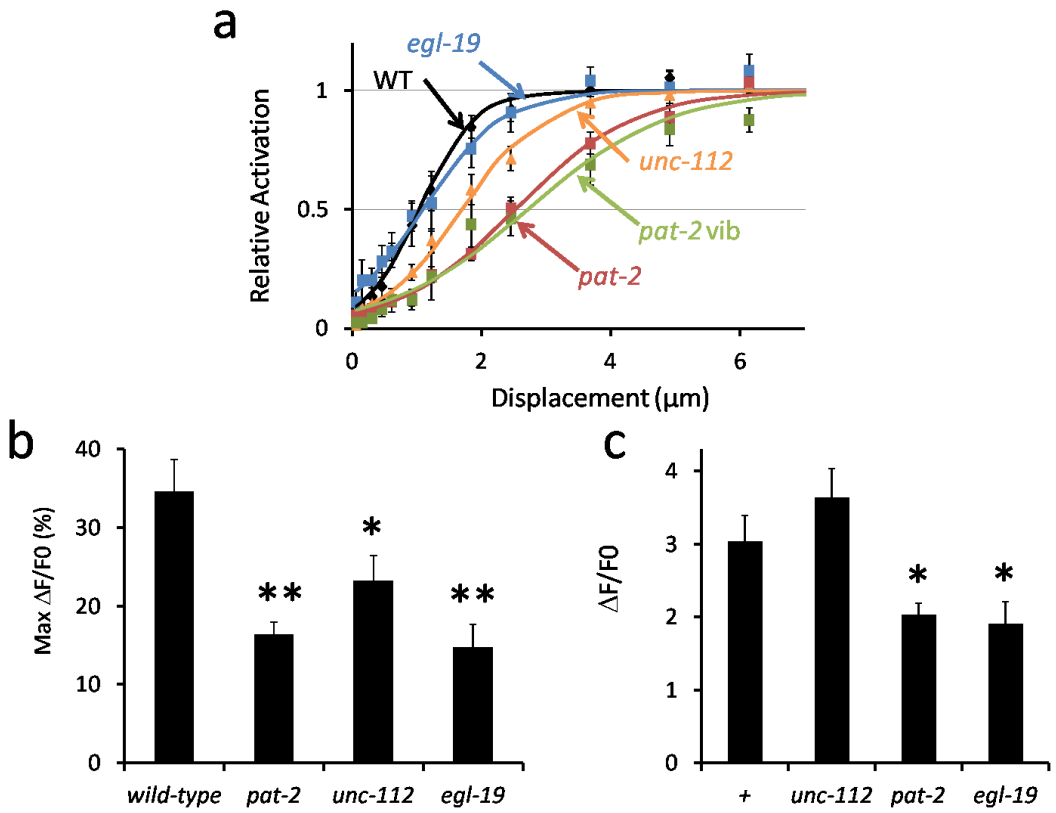

Figure 12. Calcium response of *pat-2* and *unc-112* ALM neurons. (a) Normalized calcium responses (mean ± SEM at each given force) of wild-type, *pat-2* mosaic, *pat-2* mosaic animals after sustained vibration, *unc-112* mosaic and *egl-19(ad1006)* animals under different displacements, and their corresponding Boltzmann fits. N≥6 for all strains. Wild type is reused from Figure 7b. (b) Maximum calcium responses to saturated forces from wild-type, *pat-2* mosaic and *egl-19* animals. *p=0.05, **p<0.005 using Student's t test. (c) Calcium responses (mean ± SEM) of cultured wild-type, *unc-112*, *pat-2*, and *egl-19* ALM cells to

potassium depolarization. *p=0.02 compared to wild-type response using Student's t test. N≥9 for all groups.

In addition to modulating the force response, loss of *pat-2* reduced the maximum calcium response of anterior TRNs (Figure 12b). The maximum calcium response in *unc-112* mutants was also slightly, but not significantly, reduced from that of wild type. ALM neurons in culture displayed similar differences: cells from *pat-2* mutants, but not *unc-112* mutants, showed reduced calcium responses to high potassium-induced depolarization (Figure 12c). ALM neurons from *egl-19* mutants, either *in vivo* or cultured, also showed decreased maximum calcium signal without a decrease in force sensitivity (D_{50}=1.04±0.25 µm, N=5), indicating that maximum calcium response and force sensitivity do not depend on each other. These results suggest that *pat-2* and *egl-19* induced changes in the calcium response independently of mechanotransduction and of UNC-112. Therefore, both UNC-112 and PAT-2 shifts the force sensitivity, but PAT-2 additionally modulates the calcium response.

Focal adhesion proteins signal through AKT-1 and DAF-16

Using neuronal-enhanced feeding RNAi, we screened conserved signaling genes that may cross-talk with integrin signaling (Zaidel-Bar, 2009) and found that insulin signaling and several other signaling pathways were required for optimal touch sensitivity (Table 2). Here we describe further experiments with only insulin signaling. Consistent with the RNAi results, mutations in *daf-2* (insulin receptor), *age-1* (PI3 kinase), *pdk-1* (3'-phosphoinositide-dependent kinase) and *akt-1* (AKT kinase) reduced anterior touch sensitivity (Figure 13a). None of the mutations tested affected posterior touch sensitivity except for the *daf-2(m65)* null mutation [other temperature-sensitive

daf-2 mutations, including *e979*, *m41* and *sa193*, which produce similar dauer formation and life-span phenotypes as *m65* only reduced anterior touch sensitivity (Figure 14a)].

Dense body components		Insulin-like pathway	
unc-112	Mig-2 like	*daf-2*	Insulin/IGF receptor
pat-2	α-integrin	*daf-18*	Negatively regulates *daf-2* and *age-1*
unc-97	PINCH	*age-1*	PI3k p110 catalytic subunit
pat-4	ILK	*pdk-1*	3-phosphoinositide-dependent kinase 1 ortholog
pxl-1	Paxillin	Ras/MAP kinase pathway	
pat-6	Actopaxin	*let-60*	K-RAS
nck-1	NCK adapter protein 2	*mpk-1*	MAPK
Y71G12B.11	CeTalin	*cav-1*	Caveolin
tag-224	Ortholog of LMCD1 and TESTIN	*let-92*	Protein Phosphatase 2A catalytic subunit
Rho GTPase related		*sos-1*	Son of sevenless, RAS-GEF
arf-1.1	ADP-ribosylation factor, Ras superfamily GTPase	Cytoskeleton	
H08M01.2	*rga-5*, RHO-GTPase activating protein	*F42H10.3*	Yeast homolog binds Arp2/3 (actin organization
let-502	Rho-binding ser/thr kinase ortholog (ROCK)	*tba-1*	α tubulin
unc-103	ERG-like K+ channel, activated by rho	*tsp-8*	Ortholog of KANGAI 1
unc-73	Rho-GEF for *ced-10*, *mig-2*, *rho-1*		
ced-2	SH2/3 adapter protein		
pkc-1	Ortholog to protein kinase C epsilon		
Y106G6H.14	Homolog of Yeast BEM1, which binds Cdc24p		

Table 2. Integrin signaling genes needed for mechanosensation. Each gene name is followed by a concise description.

As expected from the known pathway, loss of *daf-16* (FOXO-type transcription factor) and gain-of-function mutations in *pdk-1* and *akt-1* rescued the touch insensitivity of *daf-2*

(Figure 13b/c). The only difference between the action of these genes on touch and on dauer formation was that *akt-1* and a second AKT kinase gene, *akt-2*, are redundant for dauer formation, whereas *akt-1* was needed for anterior touch sensitivity, but *akt-2* was not (Figure 13a), even though both genes are expressed in the ALM neurons (Figure 14b). The touch insensitivity of insulin signaling mutants confirms that the activation of insulin signaling is needed for optimal touch sensitivity.

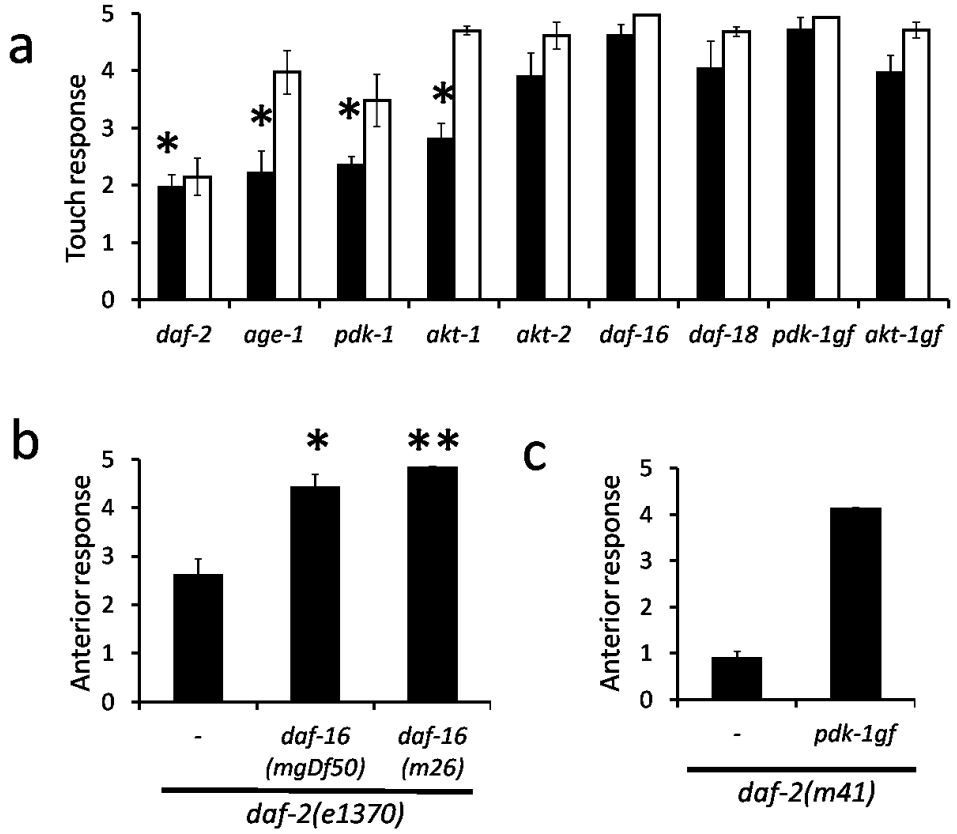

Figure 13. Touch sensitivity of insulin-signaling mutants. (a) Anterior (black) and posterior (white) response (mean ±SEM) of *daf-2(m65), sqt-1(sc13) age-1(mg109), pdk-1(sa680), akt-1(ok525), akt-2(ok393), daf-16(mgDf50), daf-18(ok480), pdk-1(mg142)* and *akt-1(mg144)*. *p<0.005 compared to *daf-16* anterior response. (b) Anterior touch response (mean ± SEM) of *daf-2(e1370)* and *daf-16(mgDf50); daf-2(e1370)* animals. *p<0.01, **p<0.0001 compared to *daf-2* alone. (c) Anterior touch response (mean ± SEM) of *daf-2(m41)* and *daf-2(m41); pdk-1(mg142)* animals. p<0.0001.

Although the touch defects of *akt-1* and *pdk-1* mutants was evident at all larval stages (data not shown), the touch insensitivity of the null *daf-2(m65)* mutants first occurred in animals 76 hrs after hatching at 15°C and was stronger after 100 hrs (Figure 14c). Since *daf-2(m65)* animals were derived from heterozygous mothers and showed maternal effects, the delay is likely to have resulted because of the presence of maternally produced DAF-2.

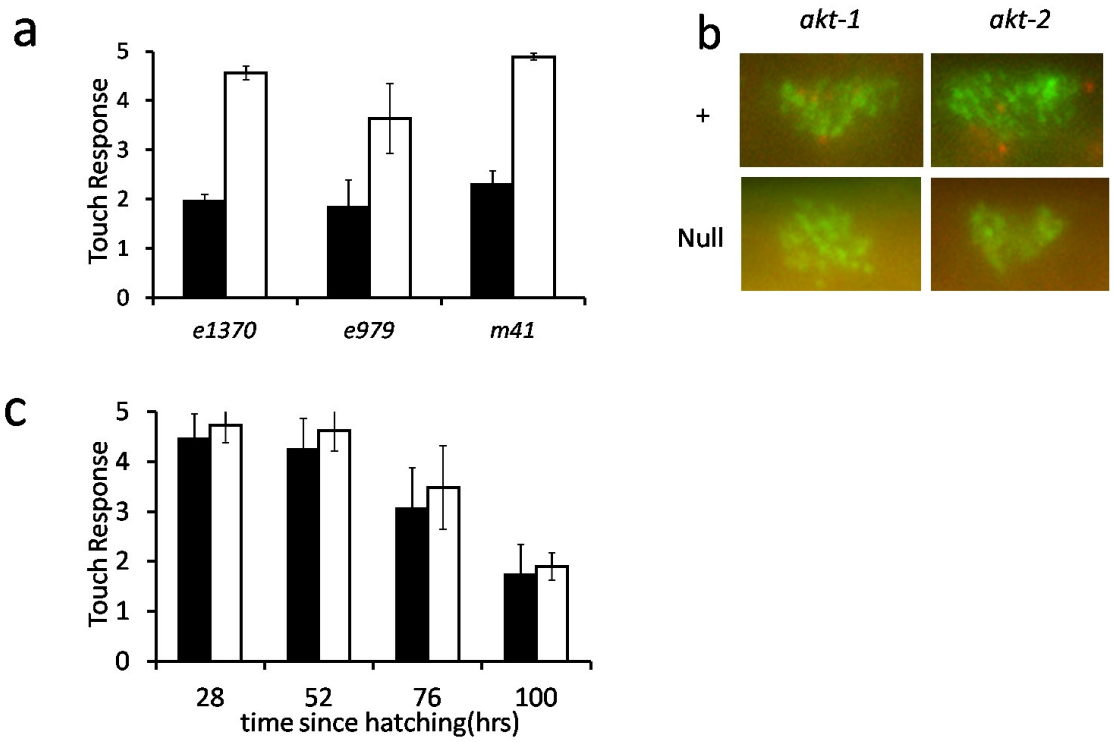

Figure 14. Touch sensitivity of additional insulin-signaling mutants. (a) Anterior (black) and posterior (white) response (mean ± SEM) of *daf-2(e1370)*, *daf-2(e979)* and *daf-2(m41)* animals. (b) Single molecule mRNA FISH of *akt-1* or *akt-2* (red), and *mec-18* (green) in wild type (+) or the respective null (null) mutants. The positions of the *mec-18* (green) dots delineate the shape of the ALM cell body. (c) Anterior (black) and posterior (white) response (mean ± SEM) of *daf-2(m65)* at the indicated stage (hours since hatching at 15°C).

The focal adhesion complex phosphorylates and activates AKT kinases in mammalian cells in culture (Persad et al., 2001). To test if integrin signaling modulated *C. elegans* touch sensitivity through AKT kinases and, thus, affected insulin signaling, we over-expressed focal adhesion genes in the TRNs in insulin signaling mutants. Over-expressing *unc-112* and/or *pat-6* in the TRNs of *daf-2(e1370)*, *daf-2(m65)*, and *pdk-1(sa680)* animals using either the *mec-17* (Figure 15a/16a/b) or *mec-3* (data not shown) promoter rescued the touch insensitivity of these mutants. No effect was seen on the posterior touch insensitivity (Figure 16b). This rescuing effect was not because of reduced expression of focal adhesion proteins in the TRNs, because *unc-112* mRNA level was unchanged by *daf-2* mutation as detected by single molecule FISH (Figure 16c). Over-expressing *unc-112* and *pat-6* in *akt-1(ok525)* null animals, however, did not rescue the anterior touch insensitivity (Figure 15a). The different effects of *unc-112* and *pat-6* over-expression on the touch sensitivity of *daf-2*, *pdk-1*, and *akt-1* animals suggest that the focal adhesion proteins act upstream of AKT-1. In support of this hypothesis, the *akt-1(mg144)* gain-of-function mutation and *daf-16(mgDf50)* loss-of-function mutation completely restored the anterior touch sensitivity to *unc-112* and/or *pat-2* mosaic animals (Figure 15b). In addition, activation of upstream insulin signaling by a *pdk-1(mg142)* gain-of-function mutation also overcame defects in integrin signaling, suggesting that integrin signaling and insulin signaling can compensate for each other with regard to touch sensitivity. Thus, AKT-1 serves as a common node coordinating these two signaling pathways. Over-expressing *unc-112* and *pat-6* or increasing insulin signaling did not enhance touch sensitivity non-specifically, because *akt-1(mg144)*, *daf-16(mgDf50)*, or over-expressing *unc-112* and *pat-6* did not rescue the touch sensitivity of

animals with the temperature-sensitive *mec-4(u45)* mutation at 23°C (Figure 16d; these mutants begin to be touch insensitive at this temperature; Gu et al., 1996).

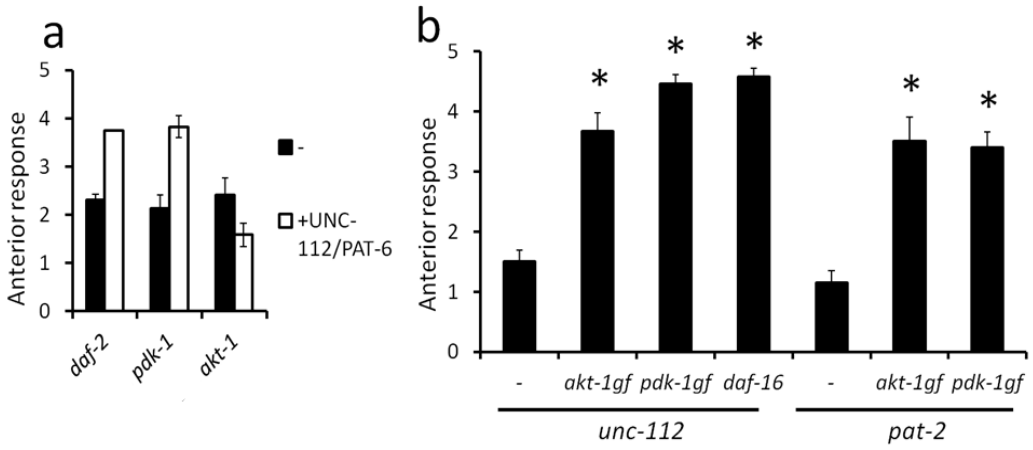

Figure 15. Interactions between insulin signaling and integrin signaling. (a) Anterior touch response (mean ± SEM of responses of individual animals) of *daf-2(m65)*, *pdk-1(sa680)* and *akt-1(ok525)* with or without *mec-17p::unc-112::gfp*, and *mec-17p::pat-6::gfp*. For *daf-2* and *pdk-1*, $p<0.001$ with or without UNC-112/PAT-6. (b) Anterior touch response (mean ± SEM of responses of individual animals) of *unc-112* or *pat-2* mosaic animals with or without *akt-1(mg144)*, *pdk-1(mg142)* or *daf-16(mgDf50)*. N>10 for all strains tested. *$p<0.0001$ compared to *unc-112* or *pat-2*.

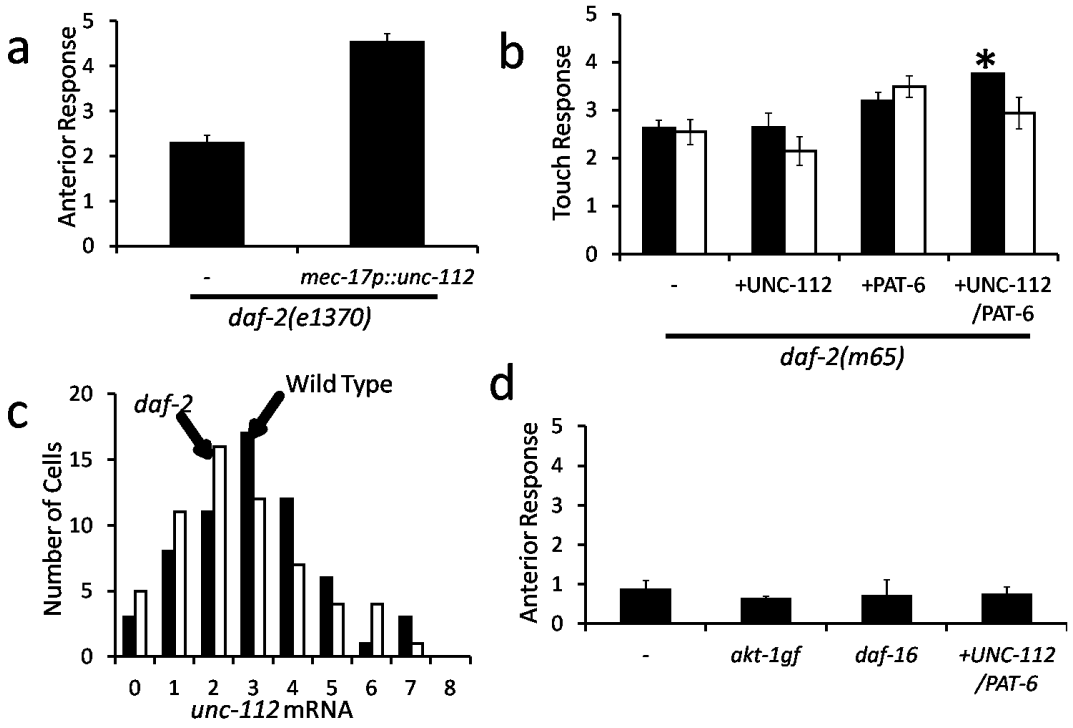

Figure 16. Touch sensitivity of animals over-expressing UNC-112 and/or PAT-6 in TRNs. (a) Anterior touch responses (mean ± SEM) of *daf-2(e1370)* with or without *mec-17p:unc-112::gfp*. $p<0.0001$. (b) Anterior (black) and posterior (white) touch responses (mean ± SEM) of *daf-2(m65)* with or without *mec-17p::pat-6::gfp*, *mec-17p::unc-112::gfp*, or both. *$p<0.015$ compared to *daf-2(m65)* anterior response. (c) Histogram of the numbers of *unc-112* transcripts per ALM cell determined by single molecule mRNA FISH. Black bars represent wild type and white bars represent *daf-2(e1370)*. No significant difference is observed between wild-type and *daf-2(e1370)* animals ($p=0.17$ using Student's t test). (d) Anterior touch response (mean ± SEM) of *mec-4(u45)*, *akt-1(mg144); mec-4(u45)*, *daf-16(mgDf50); mec-4(u45)*, and *mec-4(u45)* over-expressing UNC-112 and PAT-6 in the TRNs.

AKT-1/DAF-16 regulates MEC-4 surface expression through ubiquitination

AKT-1 modulates touch sensitivity through ubiquitination, because the anterior touch sensitivity of *akt-1* mutant animals was restored by mutations in *uba-1*, which encodes the only E1 ubiquitin activating enzyme in *C. elegans*, or *mfb-1*, which encodes

an E3 ubiquitin ligase, or by inhibiting the E1 ubiquitin activating enzyme with PYR-41 (Figure 17a). In contrast, proteosomal inhibitors, such as MG-132 and bortezomib, had no effect on the anterior touch sensitivity of *akt-1* animals (Figure 17a). The touch insensitivity of *akt-1* animals was also be restored by a mutation in *cav-1*, which encodes a caveolin and is needed for caveolae-dependent endocytosis (Lajoie and Nabi, 2010), suggesting that caveolin-dependent endocytosis may also be involved in mechanosensory modulation. *mfb-1* is expressed weakly in wild-type ALM neurons, as well as in head neurons and in the anterior and posterior ends of the gut, as shown by promoter GFP fusions and single-molecule mRNA FISH (Figure 18a/b). Consistent with the hypothesis that AKT-1 modulates touch sensitivity through MFB-1, the overall expression of *mfb-1* throughout the body and in the ALM neurons increased in *akt-1* mutants (Figure 17b). Expressing *mfb-1* or *cav-1* specifically in the TRNs of wild-type and *akt-1; cav-1* animals, respectively, reduced anterior touch sensitivity, suggesting that *mfb-1* acts cell-autonomously in the TRNs upstream of *cav-1* (Figure 17a). No such effect was seen when over-expressing *mfb-1* in the TRNs of *cav-1* animals (data not shown), or over-expressing *cav-1* in the TRNs of *daf-16* animals (Figure 17a). These data suggest that MFB-1 acts downstream of AKT-1 and reduces touch sensitivity through a caveolin dependent process.

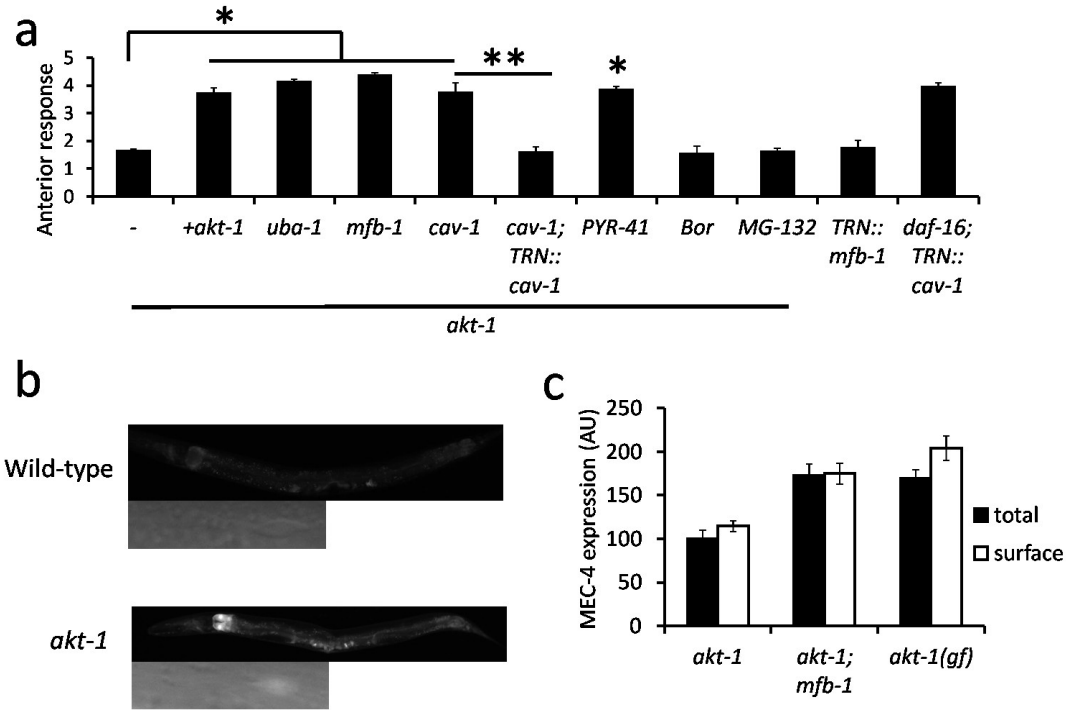

Figure 17. AKT-1 modulates touch sensitivity through MFB-1. (a) The anterior touch sensitivity (mean ± SEM) of *akt-1(ok525)* with or without *akt-1* rescuing construct, *akt-1(ok525); uba-1(it129)*, *akt-1(ok525); mfb-1(gk311)* with or without *mec-18p::mfb-1*, *akt-1(ok525); cav-1(ok2089)* with or without *mec-18p::cav-1::gfp*, *akt-1(ok525)* treated with 11 mM PYR-41, 13 μM bortezomib (Bor) or 11 μM MG-132, wild-type animals carrying *mec-18p::mfb-1*, or *daf-16* animals carrying *mec-18p::cav-1::gfp*. *p<0.0001 comparing *akt-1; +akt-1*, *akt-1; uba-1*, *akt-1; mfb-1*, *akt-1; cav-1* and PYR-41 to *akt-1* alone or *TRN::mfb-1*. **p<0.0006. (b) Expression of *mfb-1p::gfp* in wild-type and *akt-1* animals (up) and ALM neurons (down). (c) Quantification (mean ± SEM) of total (black) and surface (white) MEC-4 expression in cultured TRNs of *akt-1*, *akt-1; mfb-1* or *akt-1(gf)* animals. N≥25 for *akt-1* and *akt-1; mfb-1*, and N≥10 for *akt-1(gf)*. p<0.0001 comparing either total or surface expression of MEC-4 in *akt-1; mfb-1* or *akt-1(gf)* to that in *akt-1*.

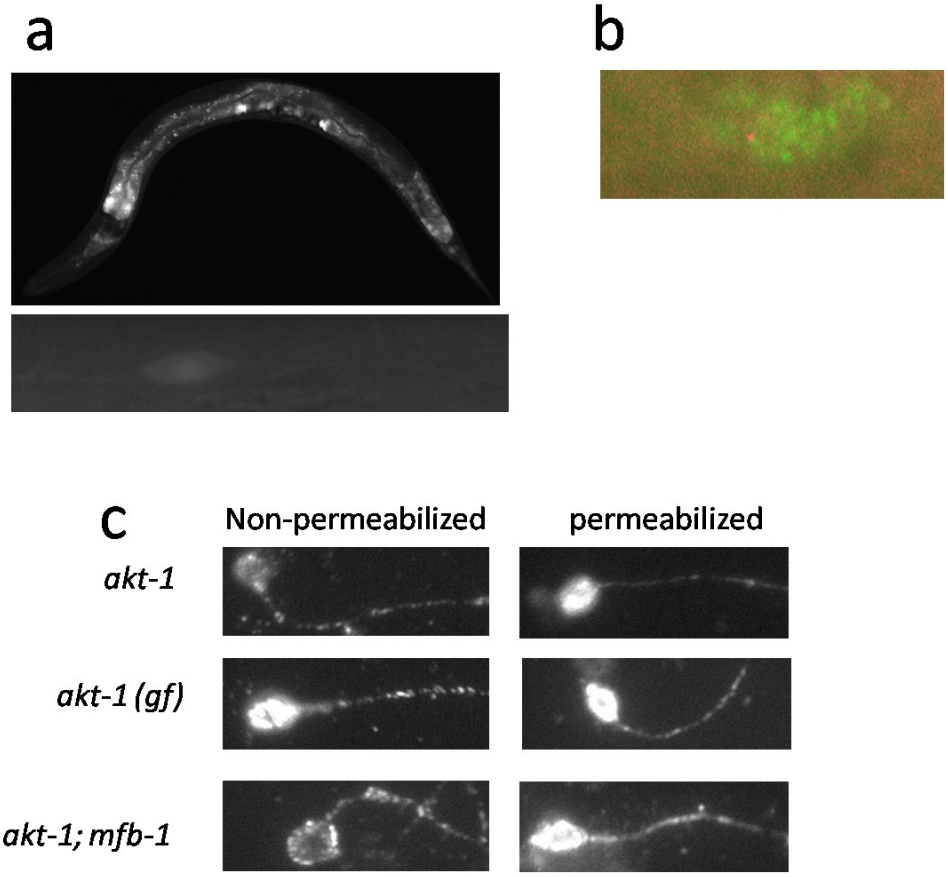

Figure 18. MFB-1 modulates MEC-4 expression. (a) *mfb-1p::gfp* expression in whole wild-type animal and in an ALM neuron in a wild-type animal fed with RNAi against *gfp*, which reduced *gfp* expression in non-neuronal tissues. (b) single-molecule mRNA FISH against *mfb-1* (red) and *mec-18* (green). (c) Antibody staining against surface (non-permeabilized) and total (permeabilized) MEC-4 in cultured TRNs from *akt-1*, *akt-1; mfb-1* and *akt-1(gf)* animals.

The mammalian ENaC channels is regulated by a caveolin-dependent endocytosis initiated by ubiquitination of the ENaC channel by the E3 ubiquitin ligase Nedd4.2 (Bhalla and Hallows, 2008), which subsequently leads to the degradation of the channel. Our data suggest that the MEC-4 channels may be regulated through a similar mechanism through MFB-1 instead of a Nedd4.2 homolog. Indeed, both surface MEC-4 expression and total MEC-4 expression were higher in TRNs carrying either *akt-1; mfb-1* or an *akt-1*

gain-of-function allele than *akt-1* loss-of-function TRNs (Figure 17c/18c), as shown by non-permeabilized and permeabilized staining of cultured TRNs. The wild-type TRNs also showed surface MEC-4 expression comparable to that of *akt-1* loss-of-function TRNs (data not shown), suggesting that insulin signaling is not activated in cultured cells, probably because secreted insulin peptides were diluted by the culture media. Therefore, AKT-1 regulates both the total and surface expression of MEC-4 through MFB-1 mediated ubiquitination.

Vibration modulates ALM neurons through integrin signaling

Integrins are activated by both ligand binding and external force (Chen, 2008), making the focal adhesions candidates for the mechanosensors responding to vibration. Indeed, growth for two hours with constant vibration restored anterior touch sensitivity to *pdk-1* and *daf-2* mutants (Figure 19a), but not in *unc-112*, *pat-2* or *akt-1* mutants, indicating that vibration compensates for loss of insulin signaling through the focal adhesion proteins and AKT-1, but not PDK-1 and DAF-2. These data suggest that the focal adhesions act as secondary mechanosensors in the ALM neurons. In support of this hypothesis, vibration did not change the force response of ALM neurons lacking *pat-2* as detected by calcium imaging (Figure 12a).

The integrins are activated by stretching force through binding to ECM components (Chen, 2008). If integrins act as secondary mechanosensors, they must bind to ECM components for the external force to be transmitted through the hypodermis to the TRNs. *him-4* mutant animals lack most of the ECM mantle around the TRN processes and the attachment between the processes and the hypodermis (Vogel and Hedgecock, 2001), and should prevent integrin activation. *him-4* animals were partially

insensitive to touch; anterior sensitivity was restored by expression of gain-of-function *pdk-1* in the TRNs (Figure 19c). *him-4* animals, however, were not sensitized by sustained vibration (Figure 19a). Because integrin signaling and insulin signaling compensate for each other in modulating touch sensitivity, these data suggest that integrin signaling cannot be activated by external force without the ECM and/or attachment to the hypodermis, further supporting our hypothesis that the integrins are mechanosensors in vibration-induced sensitization.

Vibration-induced sensitization maintains touch response under background vibration. If this sensitization occurs through the integrins and AKT-1 as we proposed, then *pdk-1* but not *akt-1* mutants should have restored touch response under sustained background vibration. Indeed, sustained vibration for over two hours increased the sensitivity to pulse under vibration in *pdk-1*, but decreased the sensitivity in *akt-1* animals (Figure 19b). The decreased sensitivity was likely caused by habituation, suggesting that vibration-induced sensitization counteracts the effect of habituation to maintain normal response under sustained vibration. These results confirm our hypothesis that vibration enhances touch sensitivity through integrin signaling and that vibration-enhanced sensitivity maintains mechanical response under background vibration.

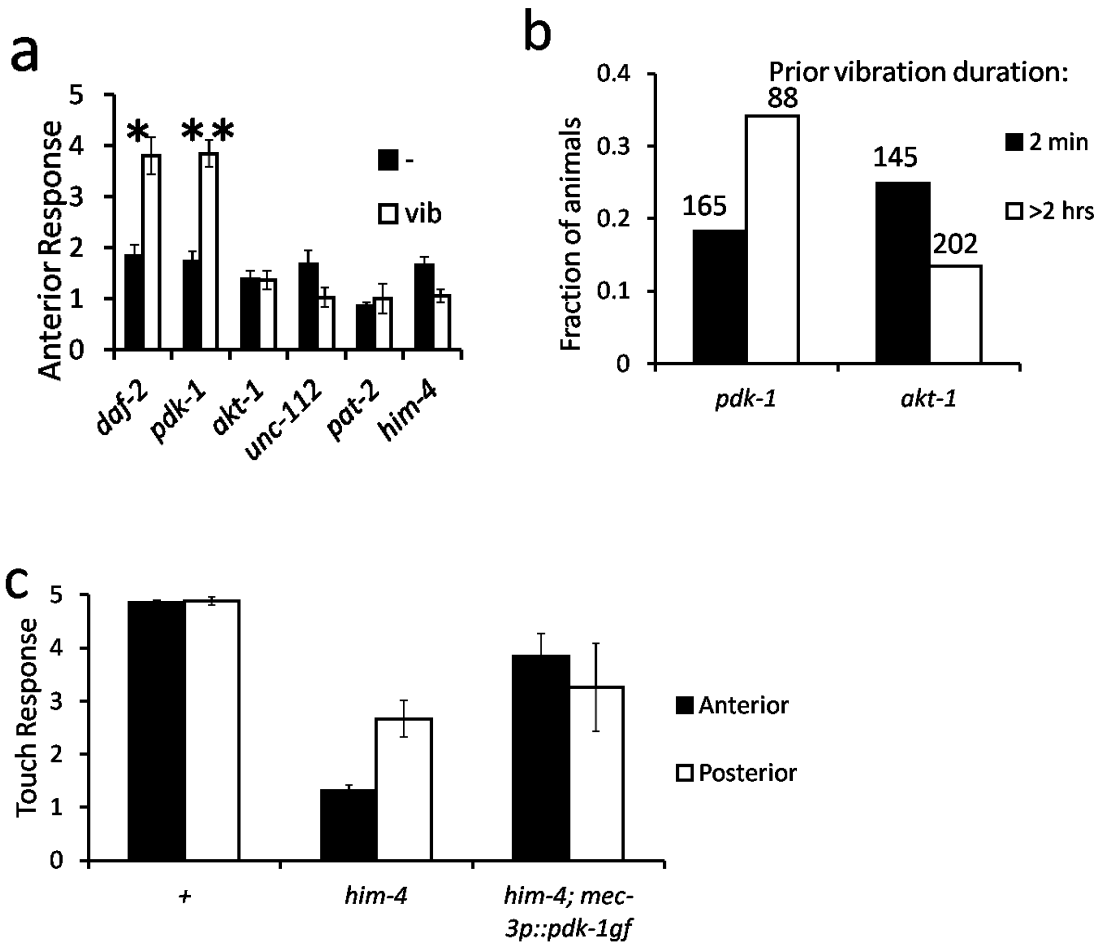

Figure 19. Touch sensitivity of insulin signaling and *him-4* mutant animals under. (a) Anterior touch sensitivity of *daf-2(d1370)*, *pdk-1(sa680)*, *akt-1(ok525)*, *unc-112 (gk1)* mosaic, *pat-2(ok2148)* and *him-4(e1267)* animals grown with (white) or without (black) vibration. *p<0.005, **p<0.0005 between vibrated and non-vibrated groups. (b) Fractions of animals moving backward in respond to pulse under background vibration with >2 hrs (white) or 2 mins (black) prior sustained vibrations. p<0.01 for both strains between with or without sustained vibrations. Total numbers of animals tested are marked on top of each bar. (c) Anterior (black) and posterior (white) touch response of wild type, *him-4(e1267)* animals, and *him-4(e1267)* animals carrying *mec-3p::pdk-1gf*. For the anterior response of *him-4*, p<0.0001 compared to wild-type anterior response and p<0.001 compared to *him-4 + mec-3p::pdk-1gf*.

Discussion

Sensory systems adapt to background stimulation levels. The sensing of gentle touch in *C. elegans* TRNs is also modified: sustained vibration increases touch sensitivity, whereas high salt decreases it. Such enhancement in touch sensitivity maintains touch sensitivity under background vibration, possibly counteracting the effect of habituation. Our results, particularly the findings that vibration, but not neuronal activity, enhanced touch sensitivity of amiloride-treated animals, indicate that the TRNs utilize integrin signaling as a secondary mechanosensory system.

Unconventional gain control of mechanosensation during sustained stimulation

Ambient stimulation modulates the gain or threshold of sensory systems. Ambient stimulation decreases sensitivity in vision (Fain et al., 2001) and olfaction (Bargmann, 2006). The gain control in *C. elegans* mechanosensation, however, differs from gain controls in these senses: an additional sensitization effect occurs after prolonged ambient stimulation in addition to habituation. A similar increase in sensitivity after sustained stimulation has been observed in mammalian hearing and touch sensing (Kujawa and Liberman, 1999; Govindaraju et al., 2006).

The effect of such sensitization counteracts habituation. When animals were given strong intermittent stimuli (1.5 s buzzes), the calcium surge that occurred in the ALM neurons in respond to the stimuli reduced significantly (Suzuki et al., 2003). In contrast, the mechanoreceptor currents from the MEC-4 channels remain unchanged during repeated stimulation (O'Hagan et al., 2005), suggesting that habituation occurs upstream of synaptic transmission within the sensory cell, but downstream of mechanosensory transduction. The difference between habituation and sensitization is that habituation occurs within minutes (Rankin et al., 1990; Suzuki et al., 2003), whereas

sensitization occurs after several hours. Such differences in timing suggest that non-linear adaptation to ambient stimulation maintains a balance. Habituation rapidly attenuates behavioral response to repeated stimuli, therefore decreasing unnecessary responses. Sensitization restores sensitivity if such stimuli persist, maintaining normal responses to mechanical stimuli. Without sensitization, as in *akt-1* animals, the animals do not respond well to strong mechanical stimuli after sustained vibration. Therefore, the combination of habituation and sensitization maintains a functional touch system under sustained ambient stimuli.

A secondary mechanosensory system modulates touch through integrin signaling

The integrins are adhesion molecules that anchor cells to the extracellular matrix. They are also mechanosensors and can be activated by force through ligand binding (Zhu et al., 2008). The activation of integrins by mechanical force induces diverse changes such as the maturation of focal adhesions (Grashoff et al., 2010) and increased cytoskeleton stiffness (Wang et al., 1993). Although changes in integrin signaling have been associated with noise exposure (Cai et al., 2012) and hyperalgesia (Dina et al., 2004), the roles of integrin signaling in neuronal mechanosensation remained unclear. We have shown that integrin signaling modulates touch sensitivity when MEC-4 channels are blocked by amiloride, suggesting that activation of the MEC-4 channel is dispensable for the modulation. This result and the finding that focal adhesion null mutants still have residual touch sensitivity suggest that the integrins and focal adhesion proteins are part of a secondary mechanosensory system that modulates the MEC-4 channel. Given the roles of integrins in cellular mechanotransduction, they may act directly as the mechanosensors in TRN sensitization.

The focal adhesion proteins also anchor the TRNs to the hypodermis, as seen by the failure of the TRN processes to separate from muscle in mutants with defects in the genes for focal adhesion proteins. This separation defect is unlikely to explain the touch insensitivity seen in these mutants since (1) both anterior and posterior TRNs failed to separate, but only anterior touch sensitivity is reduced and (2) worms treated with neuronal-enhanced RNAi against *unc-112* or *pat-6* were less touch sensitive, but had normal attachments. However, without the attachment and/or a functional extracellular matrix, it may be hard or impossible to exert force and activate the integrins. In accordance with this hypothesis, *him-4* animals, which lack extracellular matrix around the TRNs and display the TRN separation defect (Vogel and Hedgecock, 2001), cannot be sensitized by sustained vibration but can regain normal touch sensitivity through a *pdk-1* gain-of-function mutation. The importance of attachment in integrin-mediated sensitization, but not in mechanotransduction in MEC-4, suggests a different mechanism of mechanosensing through the two systems.

In addition to modulating touch sensitivity in the ALM cells by changing force sensitivity, the integrins modulate the touch-elicited calcium response. This role is independent of integrin modulation of mechanosensory transduction through AKT-1, because the calcium response caused by high potassium, which bypasses transduction, is also affected. Because the integrins act upstream of UNC-112, which does not modulate the calcium response, we propose that the integrins modulate calcium response through an alternative downstream pathway. One component of such an alternative pathway may be focal adhesion kinase (FAK), an integrin signaling factor that is not part of the ILK/PINCH/Mig-2 complex and modulates the L-type calcium channels in smooth

muscle (Hu et al., 1998). Modulation of the calcium response through FAK may also affect the touch response (although not transduction), since cytoplasmic calcium levels affect gain control in *Drosophila* mechanotransduction (Chadha and Cook, 2012).

Conserved mechanosensory modulation through integrins and AKT

The integrins are both adhesion and signaling molecules, and therefore may act either as mechanical components of mechanotransduction, or as components of signaling pathways that modulate mechanotransduction. Because gain-of-function mutations in the insulin pathway completely restored touch sensitivity in *unc-112* and *pat-2* mosaic animals, the focal adhesion proteins mainly act as signaling molecules, and are unlikely to modulate mechanotransduction directly as mechanical components.

One possible hypothesis is that insulin signaling may modulate the focal adhesion proteins, either by activating them or increasing their expression. In support of this hypothesis Suzuki and Han (2006) have reported that loss of *daf-16* or *daf-18* increased *unc-112* and *pat-6* mRNAs. Nonetheless, this hypothesis is unlikely because the *unc-112* transcription level in the ALM neurons were mostly unaffected in *daf-2* animals. In addition, such a model would predict that *unc-112* and *pat-6* over-expression should also rescue the anterior touch insensitivity of animals carrying an *akt-1* loss-of-function mutation, which we did not find. Thus, increasing *unc*-112 and *pat-6* expression can increase signaling to *akt-1* and *daf-16*, but are not likely to be the important consequence of their activity. The finding that *akt-1* gain-of-function mutation or *daf-16* loss-of-function mutation rescued the touch insensitivity of *unc-112* and *pat-2* mosaic animals further suggests that the integrins and focal adhesion proteins act upstream of AKT-1 and DAF-16, in parallel with the upstream insulin signaling.

We suggest that integrins sense external forces and activate AKT-1 through the focal adhesion proteins. AKT-1 then modulates touch sensitivity through transcriptional regulation of *mfb-1*, which encodes an E3 ubiquitin ligase that regulates MEC-4 surface expression. In mammals, ubiquitination of mammalian ENaC channels by the E3 ubiquitin ligase Nedd4.2 induces endocytosis and degradation of the channel (Bhalla and Hallows, 2008; Kabra et al., 2008), thus changing the amount of function channel on the plasma membrane. Although MEC-4 lacks the conserved recognition sites of Nedd4.2, our data suggest that similar modulation occurs on the MEC-4 channels through a different E3 ligase, MFB-1. More surface MEC-4 increases TRN depolarization by force, thus increasing the TRN sensitivity to force. This hypothesis would predict that when surface MEC-4 expression is changed, the slope of the force response curve would also change. Our calcium response data of the *unc-112* mutants, *pat-2* mutants, and sensitized wild-type animals are consistent with this model.

In mice, integrin signaling through the AKT kinase, FOXO transcription factors and Atrogin-1, the mouse homolog of MFB-1, contributes to muscle hypertrophy following exercises (Sandri et al., 2004; Marino et al., 2008; Zou et al., 2011). Although we do not have definitive proof that AKT-1 regulates MFB-1 through DAF-16 in *C. elegans*, two evidences suggest that DAF-16 is involved. Three forkhead transcription factor recognition sites (TRTTTAY) are present at ~800-900bp upstream of the start codon of *mfb-1* and one additional site ~110bp upstream of the start codon of *mfb-1*. In addition, both positions are bound specifically by DAF-16 as shown by the ModEncode project (Celniker et al., 2009). These observations suggest that integrin signaling through AKT-1 could be a conserved modulatory pathway activated by mechanical force.

Integrin signaling also plays diverse roles in other mechanosensory systems. Blocking integrin signaling with RGD-containing peptides, antibodies or RNAi against α1, α3, α5 or β1integrins, for example, restored mechanical pain threshold reduced by inflammatory mediators (hyperalgesia) (Dina et al., 2004), suggesting that increased integrin signaling also increases the sensitivity of mechanical nociception. In the vertebrate hair cells, mutations of integrins or the integrin-binding protein CIB2 cause deafness (Littlewood Evans and Muller, 2000; Riazuddin et al., 2012). CIB2 is localized at the tip of stereocilia and may be involved in calcium regulation of the stereocilia. Mutations in these two genes also cause developmental defects of the stereocilia, suggesting that similar to the TRNs, integrin signaling may also affect both the development and the function of the hair cells. Both hearing and mechanical nociception can be sensitized through repeated stimuli in rodents and/or human (Kujawa and Liberman, 1999; Govindaraju et al., 2006; Chen et al., 2010), leading to the speculation that the long-term sensitization we observed in *C. elegans* may be a conserved and common characteristic of mechanosensory systems.

Experimental procedures

Strains

C. elegans strains (see Appendix II for details) were maintained at 15°C or 20°C as described by Brenner (1974). Temperature-sensitive strains were maintained at 15°C and transferred to 25°C for one generation before testing.

Most strains were provided by the Caenorhabditis Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *pat-2(ok2148) III, unc-112(gk1) V, akt-1(ok525) V* and *akt-2(ok393) X* were generated by the International *C. elegans* Gene Knockout Consortium (http://www.celeganskoconsortium.omrf.org). *pat-2* translational GFP fusion (JE2222) was a gift from Dr. Jean Schwarzbauer.

TU3842 (*uIs109 III; him-5(e1490) V*) was made by injecting *ceh-22p::gfp* and *mec-17p::gcamp3* to generate an integrated insertion *uIs109* in CB1490 (*him-5(e1490) V*). We then crossed TU3842 into *egl-19(ad1006) IV*, *mec-4(u253) X*, and *pat-2* and *unc-112* mosaic strains to produce the GCaMP3 strains in the corresponding backgrounds.

TU3595 (*sid-1(pk3321) him-5(e1490) V; lin-15b(n744) X; uIs72*) was made by injecting *unc-119p::sid-1*, pCFJ90 and *mec-18p::mec-18::gfp* to produce an integrated insertion *uIs72* in NL3321 (*sid-1(pk3321) V*) and then crossed into TU3596 (*sid-1(pk3321) him-5(e1490) V; lin-15b(n744) X*). *lin-15b(n744)* was confirmed by PCR and sequencing.

For GFP fusions of focal adhesion genes, we co-injected the GFP expression plasmids with pCFJ90 (*myo-2p::mCherry*; Addgene, Cambridge, MA) as injection markers to generate extrachromosomal arrays.

To produce focal adhesion gene mosaics, we generated transgenic animals by microinjecting the corresponding rescuing constructs with pCFJ90 and *mec-3p::rfp*, and isolated animals homozygous for the mutations in focal adhesion genes by picking animals whose progeny all have the extrachromosomal array (non-transgenic animals would be dead as embryos).

To generate the channelrhodopsin strains, we injected *mec-4p::chr2::yfp, mec-17p::gfp* and pCFJ104 to generate integrated insertions *uIs91* and *uIs94* in TU3403 (*ccIs4251 I*; *sid-1(qt2) V*; *uIs71*) (Calixto et al., 2010a) to produce TU3597 and TU3600. We then crossed *uIs91* into *egl-19*, *unc-97*, *unc-112* and *pat-6* animals and *uIs94* into *pat-2* and *pat-3* animals to produce the channelrhodopsin strains in each background.

Constructs

mec-17p::unc-97::gfp, *mec-17p::pat-6::gfp* and *pat-2p::gfp* were made by inserting digested PCR amplified products into pPD95.75. pCFJ90 (*myo-2p::mCherry*) and pCFJ104 (*myo-3p::mCherry*) were obtained from Addgene (Frokjaer-Jensen et al., 2008). *mec-4p::chr2::yfp* (Nagel et al., 2005) was a gift from Dr. Alexander Gottschalk. All other constructs were made using the three-fragment Gateway system (Life Technologies, Grand Island, NY). The promoters and genomic coding regions were cloned into pDONRP4-P1r and pDONR221, and the *unc-54* 3'UTR with or without *gfp* or *rfp* were cloned into pDONR P2r-P3. The three plasmids containing the promoter, the coding region and the 3'UTR were then recombined into pDEST R4-R3 to make the final rescuing constructs. Primers used are listed in Appendix II.

Mosaic analysis

Rescued *pat-2, pat-3, pat-6, unc-97 and unc-112* strains all have *mec-3p::rfp* in the extrachromosomal array as a TRN marker. Animals were grown to the L4 stage and observed under a Leica M12 stereoscope modified with a M2 Bio Quad fluorescent attachment (Kramer Scientific LLC, Amesbury, MA) and an EXFO X-cite 120 metal halide light source (EXFO, Quebec, Canada). Animals lacking RFP in ALM and AVM cells, the two PLM cells, or having RFP in all six TRNs (controls) were selected and scored blindly for both anterior and posterior touch sensitivity. After the whole test was completed, the anterior touch response of animals that had lost the rescuing array in the anterior TRNs, and the posterior touch response of animals that had lost the rescuing array in the posterior TRNs were compared to both the anterior and posterior touch response of the controls. For calcium imaging experiments, the absence of RFP in the ALM cells was additionally confirmed under a 40× 0.95 lens. For all experiments involving mosaic animals, at least two independent tests were done and the data were pooled together for analysis.

Feeding RNAi

The feeding RNAi screen was performed with minor differences as described by Calixto et al. (2010a) using TU3595. Animals were grown on 6-well plates instead of 5 cm dishes. RNAi against each gene was tested four times independently, and genes that produced touch insensitivity in three out of four tests were considered as causing touch insensitivity. For a single test, we estimated the false positive rate to be <20% from a larger screen for ~1000 genes. Therefore, the false positive rate of the screen was

estimated to be $C_4^1 \times 0.2^3 \times 0.8 + 0.2^4 = 2.7\%$, corresponding to $2.7\% \times 80 = 2$ false positives out of the 23 genes obtained from the screen. Subsequent tests of all identified insulin and Ras/MAPK pathway genes using mutant alleles identified *cav-1* and *daf-18* as the only two false positive in these two pathways.

As a control we included the seven dense body genes found in our initial study (Calixto et al., 2010a) in our list of 87 candidate genes, and were able to identify six of them blindly (the remaining gene, *pat-3*, produced lethality in TU3595). RNAi for 23 of the remaining 80 genes reduced touch insensitivity (Table S1). Based on known functions or homology, the 23 genes included additional focal adhesion genes, genes in the Ras/MAPK pathway, insulin signaling pathway, Rho-GTPases related genes and cytoskeleton related genes. *unc-73*, which encodes a guanine exchange factor for several Rho-GTPases, and *mec-12*, which is a secondary target for *tba-1* RNAi, were previously reported to affect TRN functions and/or development (Hedgecock et al., 1987; Chalfie and Thomson, 1982).

Touch sensitivity assay

We tested touch sensitivity using a method modified based on the one described by Chalfie and Sulston (1981). Instead of touching the animal by sliding a thin hair across the top of the worm, we gently touched the side of the animal. For *akt-1(ok525)* animals, five anterior touches produced on average 4.4 ± 0.2 responses (mean ± SEM of responses of individual animals, N=20) by slashing a thin hair across the animal, and 1.7 ± 0.2 (N=19) by touching the side of the animal. Both methods produced similar scores

for wild-type animals (4.9 ± 0.1 for original method and 4.6 ± 0.1 for side touch, N=16 and N=18, respectively).

Each animal was tested five times anteriorly and five times posteriorly unless noted (Hobert et al., 1999). 10 to 20 animals were tested each time, and each strain was tested at least three times independently. The average and SEM of the means from the independent tests were reported unless noted, with the exception of mosaic analysis, in which the average and SEM of the response of animals were reported. p-values were calculated using Student's t test.

For drug treatment on *akt-1* animals, the animals were grown on agar plates with the specified drugs dissolved in the agar for at least 24 hrs before testing.

Channelrhodopsin assay

Bleached eggs were put on plates seeded with 100 µl *E. coli* OP50 in LB broth at the stationary phase (OD_{595}=0.45; Brenner, 1974) with 500 µM all *trans*-retinal (Sigma-Aldrich Co., St. Louis, MO). Animals grown for five days at 15°C were tested for touch sensitivity as young adults under a Leica M12 stereoscope modified with a M2 Bio Quad fluorescent attachment (Kramer Scientific LLC, Amesbury, MA) and an EXFO X-cite 120 metal halide light source (EXFO, Quebec, Canada). We then exposed each worm to a <0.5 s flash of blue light coming from the GFP filter under a 20× f=0.6 objective and 12× zoom in the region between the nerve ring and the vulva. A fast backward movement within the 0.5 s exposure was counted as a positive response. Each animal was tested three times instead of five times because considerable habituation occurs after three tests (Nagel et al., 2005). p-values were calculated using Student's t test.

Microscopy and quantification of puncta.

All images were taken on a Zeiss Axioskop with a SPOT-2 slider camera (SPOT Imaging Solutions, Sterling Heights, MI) or a Zeiss Observer Z1 microscope with a Photometrics CoolSnap HQ^2 camera (Photometrics, Tucson, AZ). When we wanted to quantify results we blinded the samples and used ImageJ to score traits (Schneider et al., 2012).

A TRN process was scored as attachment defective if it was close to the muscle for more than half its length when examined under 63× magnification. For RNAi treated animals (Figure 11c), an ALM process was scored as attachment defective if the ALM cell body was squeezed into a half-circle shape instead of the normal raindrop shape. We scored an ALM cell as migration defective when the cell body was anterior either to the mid-point between terminal bulb of the pharynx and the vulva, or to the AVM cell body. p-values were calculated using Fisher's exact test.

We performed single-molecule mRNA FISH as described by Topalidou et al. (2011).

Calcium Imaging

Calcium imaging was performed as described in Chapter II. The displacement of the cuticle of the animal is calculated by recording the movement of the probe movement under DIC illumination at the voltages used for calcium imaging. The voltage – displacement relation obtained from multiple animals was then fitted with a linear model to obtain the average displacement at each voltage. Differences in displacement among

different animals are small (<10%). Therefore the average values of displacement were then used as the probe displacement occurred during the calcium recordings at corresponding voltage settings.

Only late L4 larvae or young adult animals were used for calcium imaging, although L3 larvae had similar touch sensitivity and calcium response (data not shown). For a typical experiment, we touched an animal with increasingly larger voltage from 0.01 V to 1.6 V, once or twice at each voltage, and recorded the calcium signal. We then subtract a blank background from the average intensity of the cell body to obtain the peak calcium response at each voltage. The calcium response was normalized to the maximum calcium response for each animal and fitted with a Boltzmann equation using Solver in Microsoft Excel. p-values for D_{50} and maximum calcium response were calculated using Student's t test.

Calcium imaging of TRNs cultured on coverslips (Topalidou and Chalfie, 2011) was performed according to Suzuki et al. (2003). *pat-2* or *unc-112* ALM cells were selected by finding GCaMP3-positive and RFP-negative cells with one long process only. ALM cells from other strains were selected by finding GCaMP3-positive cells with one long process only.

Vibration assay

We played wav files containing the appropriate waveforms through 3.5" (for culture) or 5.25" (for observation) dual cone speakers amplified by a digital amplifier. The outer cones of the speakers were cutout, leaving only the inner cone as a stand for the plates. A lid of a 3.5 cm petri dish (BD Falcon 351008) was glued to the top of the cone

facing up, and the bottom of the dish was glued to a test plate, also facing up. The test plate was then put on the speaker by fitting the dish bottom to the lid, which produce enough friction to hold the plate in place. The wav files were written with Matlab (Mathworks, Natick, MA). The peak acceleration of the vibration was quantified using a DE-ACCM6G accelerometer (Dimension Engineering LLC, Akron, OH) on top of the plates. To observe the plates under vibration, we illuminated the plates on one side from underneath and recorded with a HD video-capable camera.

For all touch tests regarding vibration, the animals were vibrated with 50 Hz square waves for 24 hours with a peak acceleration of 1.5 g, and recovered from habituation for 30-40 min before testing unless otherwise noted. For high-salt treatments, the animals were grown on NGM plates (Brenner, 1974) supplemented with an additional 180 mM NaCl for one generation. For amiloride treatment, *bus-17* animals, which are more drug permeable as adults (Gravato-Nobre et al., 2005) were transferred to NGM plates supplemented with 2 mM amiloride hydrochloride hydrate (Sigma-Aldrich Co., St. Louis, MO) and 180 mM NaCl for at least two hrs, vibrated for two hrs, and recovered for one hr on hyperosmotic NGM plates without amiloride before testing. For channelrhodopsin activation during culture, TU3851 animals were grown on hyperosmotic plates until the L4 stage, transferred to a hyperosmotic plate wrapped in aluminum foil and illuminated by a LXML-PB01-0040 Luxeon Rebel 470 nm LED (Philips Lighting US, Somerset, NJ) at 700 mA for two hours, then rested for 30-40 min before testing. For calcium imaging experiments, wild-type animals were vibrated for 24 hrs at 0.7 g peak acceleration and tested immediately without recovery.

To test touch sensitivity during background vibration, we inserted a 0.5 s 0 dB 50 Hz square wave pulse in a background of -4 dB 50 Hz square wave vibration, reaching peak accelerations of 1.7 g and 0.7 g in the plates, respectively. We videotaped the animals and scored them on the videos.

Antibody staining

Cultured cells were fixed with 4% PFA in BSA for 10 min at room temperature, and blocked in 1% BSA in PBS with or without 0.5% Triton for 30 min at 4˚C. The slides were then washed in PBS, incubated in PBS with primary antibodies for 1 hr at 4˚C, then washed overnight before incubation with the secondary antibodies for 1 hr at 4˚C. The slides were then washed four times and mounted for microscopy.

Chapter IV Contextual modulation of gentle touch sensitivity in *C. elegans* through insulin-like peptides

Abstract

Sensory perception is modulated by the environment, an animal's internal state and other sensory modalities. Modulation ensures that sensory inputs generate the proper behavior response under diverse conditions. However, how these factors are integrated to modulate sensory perception is unclear. Previously we have shown that integrin signaling acting upstream of AKT-1 modulates anterior touch sensitivity in *C. elegans* after sustained mechanical stimuli. In this paper, we show that INS-10, an insulin-like peptide, modulates anterior touch sensitivity as a long-range neuromodulator through the canonical insulin signaling pathway. INS-10 expression decreases under several stressful conditions such as dauer formation and hypoxia. Reduction of INS-10 attenuates mechanosensory responses, but increases efficiency in other tasks, such as chemotaxis, when mechanical stimuli are also present. We hypothesize that the reduced touch sensitivity under these conditions allows animals to be focused on escaping the current conditions with less distraction from mechanical stimuli, thus increasing their chances of survival. The signaling downstream of INS-10 converges with integrin signaling at AKT-1 and MFB-1, and increased integrin signaling compensates for loss of INS-10 in terms of mechanosensory modulation. These findings suggest that AKT-1 and MFB-1 are a key modulatory mechanism of anterior touch sensitivity capable of integrating diverse contextual information.

Introduction

Context modulates sensory perception under diverse conditions that change an animal's behavior to sensory stimuli. For example, a songbird's response to songs is dependent on estradiol levels, which fluctuates seasonally (Maney and Pinaud, 2011), and in the nematode *C. elegans*, gustatory chemosensation can be enhanced under hypoxic conditions by recruiting additional neurons into the circuit (Pocock and Hobert, 2010). Although some of these effects occur at the circuit level, sensory cells can also be modulated directly. For example, mammalian cone and rod cells adjust their dynamic range in response to the overall brightness (Fain et al., 2001). Sensory modulation can act through direct synaptic connections (Wersinger and Fuchs, 2010), long-range neuropeptides (London et al., 2009) or hormones (Niki et al., 2010; Page et al., 2009).

Mechanosensation in particular can habituate or sensitize upon previous experiences (Rankin et al., 1990; Kujawa and Liberman, 1999), be modulated by visual perception or other forms of mechanosensation such as hearing (Longo et al., 2011;Hotting et al., 2003), or be inhibited by motor functions (Cattaert et al., 2002;Torkkeli and Panek, 2002). Environmental factors such as the availability of food (Kindt et al., 2007) also modulate the habituation rate of mechanosensation, but not mechanosensation itself. Whether and how environmental factors directly modulate the sensitivity of the mechanosensory cells is unclear.

In this paper, we investigate how multiple environmental and internal factors modulate sensitivity to gentle touch in *C. elegans*. The six touch receptor neurons (TRNs) in *C. elegans* sense gentle touch along the body by the DEG/ENaC MEC-4/MEC-10 (O'Hagan et al., 2005) mechanotransduction channels. In addition to the

MEC-4 and MEC-10 channel subunits, these channels require several other membrane proteins, including MEC-6 and MEC-2, both of which are essential for touch sensitivity (Chelur et al., 2002;Goodman et al., 2002). Additional cytoplasmic proteins and extracellular-matrix components are also needed for the function and the placement of the mechanotransduction channels (Savage et al., 1989; Bounoutas et al., 2009ba; Bounoutas et al., 2009ab; Topalidou et al., 2012; Emtage et al., 2004).

In addition to these proteins essential for touch sensitivity, we have previously shown that the integrins and focal adhesion proteins also affect touch sensitivity (Calixto et al., 2010a). Instead of being essential components of mechanosensation, the integrins and focal adhesion proteins are part of a secondary mechanosensory system that modulates the force sensitivity of the MEC-4/MEC-10 channels under vibration. The focal adhesion proteins signal through downstream components of the canonical insulin signaling, including AKT-1 and DAF-16 (Hu, 2007), to regulate the transcription of MFB-1, an E3 ubiquitin ligase that regulates the membrane trafficking of MEC-4. This signaling convergence suggests the interesting possibility that additional factors may also modulate touch sensitivity through the canonical insulin signaling.

Insulin signaling can be activated by insulin-like peptides. Insulin-like peptides modulate a wide range of neuronal processes, such as self-control (Gailliot et al., 2007), decision-making (Wang and Dvorak, 2010) and memory consolidation (Chen et al., 2011), in addition to their traditional roles of regulating blood glucose as a hormone. In *C. elegans*, 40 genes encode insulin-like peptides that regulate diverse processes. INS-1, INS-4, INS-6, INS-7 and DAF-28 regulate dauer formation (Murphy et al., 2003; Li et al., 2003; Cornils et al., 2011); INS-33 is needed for larval development (Hristova et al.,

2005); INS-1 modulates learning (Lin et al., 2010); INS-7 and DAF-28 are involved in the innate immune response (Hahm et al., 2011; Kawli and Tan, 2008); and INS-7 and INS-11 modulate longevity (Murphy et al., 2003; Kawano et al., 2006). The functions of other insulin-like peptides remain largely unknown.

Here we show that multiple stressful environmental (hypoxia) and internal conditions (dauer formation) modulate sensitivity to gentle touch in *C. elegans* through an insulin-like peptide, INS-10, which acts as a long-range hormonal neuropeptide. Decreased INS-10 level reduces anterior touch sensitivity specifically by changing the force sensitivity through insulin signaling and AKT-1, but not downstream calcium responses in the ALM neurons. Animals with decreased INS-10 level, however, become more efficient at tasks such as chemotaxis under vibration, suggesting that animals de-prioritize touch response under stressful conditions to facilitate escape. Because both integrin signaling and insulin signaling converge on AKT-1 to modulate touch sensitivity, we propose that AKT signaling is a master modulatory pathway that integrates diverse mechanical (through integrin signaling) and non-mechanical (through insulin-like peptides) information into mechanosensory modulation.

Results

Multiple insulin-like peptides are needed for optimal touch sensation

40 *C. elegans* genes encode insulin-like peptides (Pierce et al., 2001). We tested touch sensitivity in mutants with loss-of-function alleles in 32 of these genes and RNAi-fed animals for the remaining genes (*ins-10*, *ins-19*, *ins-20*, *ins-21*, *ins-24*, *ins-32*, *ins-36*, *ins-37*, and *ins-39*). Mutation or reduction of *ins-10*, *ins-22*, *ins-33* and *ins-37* caused anterior touch insensitivity (Figure 20a). INS-33, which is needed for larva development (Hristova et al., 2005), probably acts generally, since *ins-33* larvae that had normal growth were touch sensitive (data not shown). A strain (RB2059) carrying *ins-28(ok2722)* was also touch insensitive initially (data not shown), but was touch sensitive after outcross, suggesting that the touch insensitivity was caused by background mutations.

INS-10 and INS-22 appeared to regulate touch sensitivity, since animals carrying *ins-10(tm3498)*, *ins-22(tm4990)*, or a construct expressing an *ins-10* hairpin under *ins-10* promoter to induce RNAi against *ins-10* (*ins-10(i)*) had apparently normal development and were not dauer-constitutive or dauer-deficient (data not shown). RNAi against *ins-10* in an *rde-1* background, which abolishes RNAi, had no effect on touch sensitivity. Expressing a wild-type *ins-10* with alternative codons in *ins-10* RNAi animals, or expressing a wild-type copy of *ins-10* or *ins-22* in *ins-10(tm3498)* or *ins-22(tm4990)* animals, restored the touch sensitivity to *ins-10* RNAi animals (Figure 20a). We used *ins-10* RNAi animals for most subsequent analyses because the putative deletion allele, *ins-10(tm3498)* produced detectable *ins-10* mRNA as detected by both rtPCR and single molecule mRNA FISH (J. Alcedo, personal communication; Figure 20b).

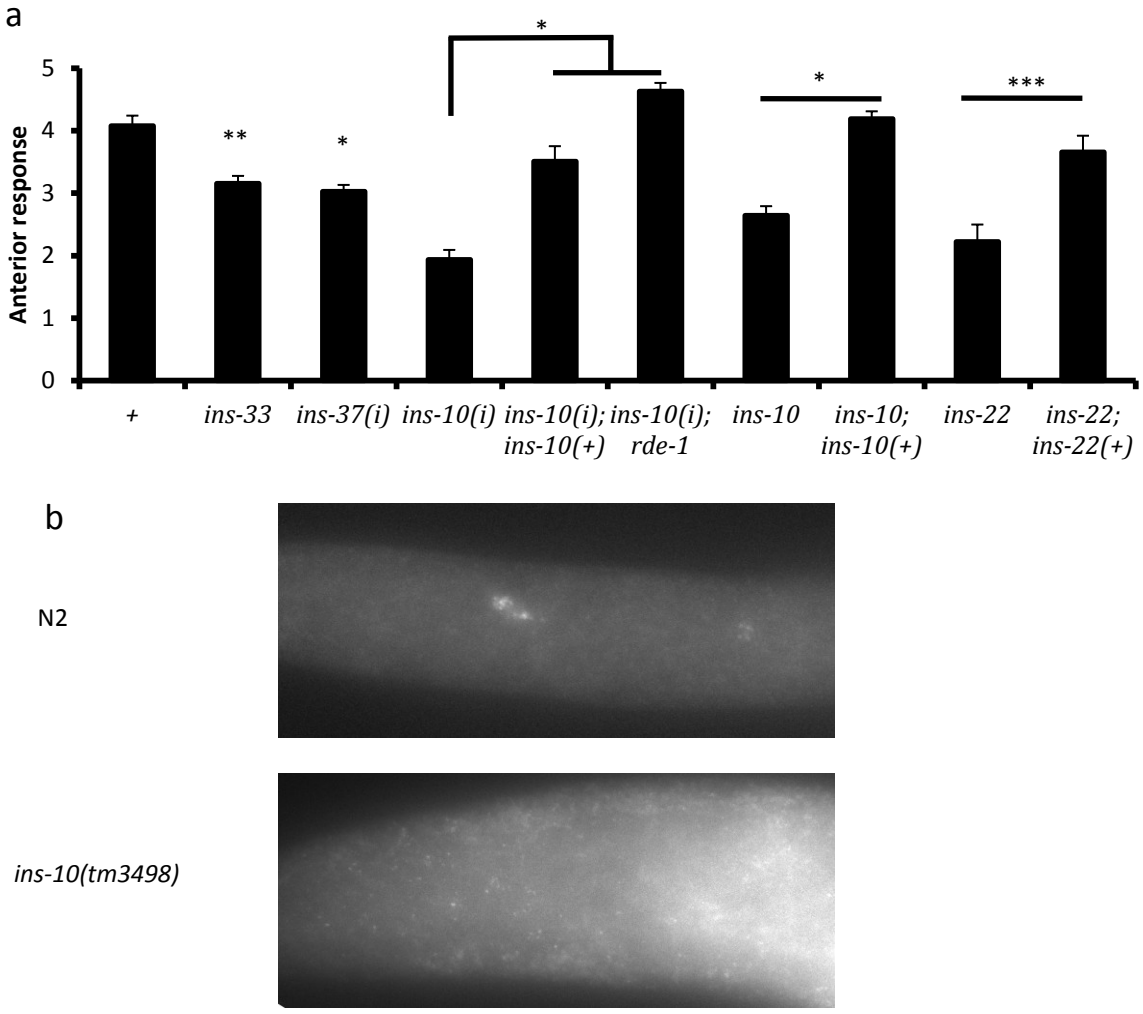

Figure 20. Anterior touch sensitivity of insulin-like peptide deficient animals. (a) the anterior touch sensitivity (mean ± SEM) of animals carrying *ins-33(tm2988)*, TU3595 animals treated with feeding RNAi against *ins*-37, wild-type animals expressing an RNAi construct against *ins-10* (*ins-10(i)*) alone, *ins-10(i)* animals carrying either a wild-type copy of *ins-10* with different codons or an *rde-1* mutation, *ins-10(tm3498)* animals with or without a wild-type copy of *ins-10*, and *ins-22(tm4990)* animals with or without wild-type copies of *ins-22*. N>3 for all strains tested. *p<0.0001, **p<0.01, ***p<0.02. (b) single molecule mRNA FISH against *ins-10* transcripts in N2 and *ins-10(tm3498)*. See also Figure 22.

INS-10 acts through the canonical insulin-signaling pathway in the TRNs

To determine how INS-10 modulates touch sensitivity, we performed calcium imaging in ALM neurons expressing GCaMP3, and found that *ins-10* RNAi animals had reduced force sensitivity compared to wild-type animals, as measured by the probe displacement required for 50% activation (D_{50}=2.27±0.49 μm for *ins-10(i)*, N=11, $p<0.02$; D_{50}=1.11±0.12 μm for N2, N=11; Figure 21a), but normal maximal calcium response (Figure 21b). This modulation is similar to the focal adhesion-dependent modulation of touch sensitivity, which acts in parallel with insulin signaling and converges on AKT-1.

The touch insensitivity caused by *ins-10* RNAi was suppressed in animals with the *pdk-1(mg142)* or *akt-1(mg144)* gain-of-function alleles or with the equivalent construct of *pdk-1(mg142)* expressed only in the TRNs (Figure 21c). These results suggest that INS-10 acts through canonical insulin signaling in the TRNs. Loss-of-function mutations in genes for other components of the pathway (the insulin receptor DAF-2, the PI3-kinase AGE-1, and the AKT-1 kinase) all produced a similar defect to *ins-10* RNAi. In addition, over-expressing UNC-112 (Mig-2) and PAT-6 (Actopaxin) in the TRNs rescued the touch insensitivity caused by *ins-10* RNAi (Figure 21c). These two proteins are core components of focal adhesions and their over-expression has been shown to compensate for defects in the insulin-signaling pathway. We also found that long-term vibration, which activates integrin signaling and may compensate for reduced insulin signaling, also rescued the anterior touch sensitivity of *ins-10* RNAi animals (Figure 21c). Given these results, INS-10 affects anterior touch sensitivity through the canonical insulin signaling in the TRNs. These results also confirm our previous

observations that integrin signaling and insulin signaling compensate for each other for touch sensitivity.

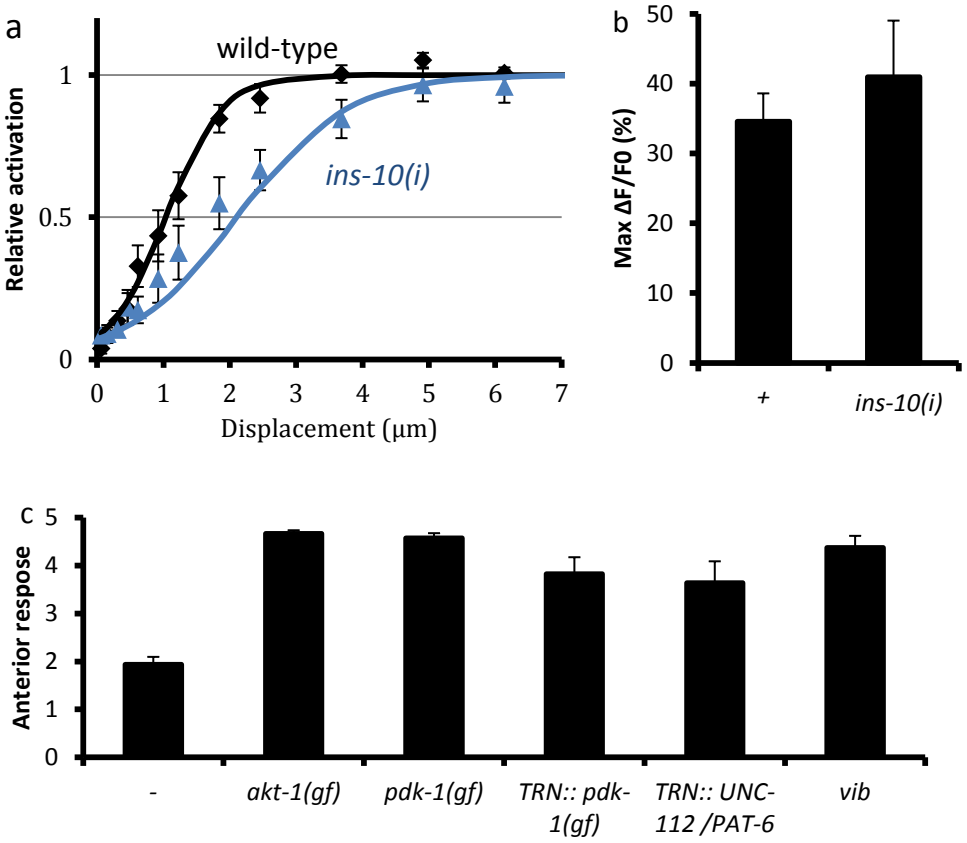

Figure 21. INS-10 modulates touch sensitivity through insulin signaling. (a) Normalized calcium responses (mean ± SEM) of wild-type (black) and *ins-10(i)* (blue) animals under different probe displacements, and their corresponding Boltzmann fits. N=11 for both strains. (b) Maximum calcium responses (mean ± SEM) to saturated forces in wild-type and *ins-10(i)* ALM neurons. (c) Anterior touch response (mean ± SEM) of *ins-10(i)* animals with or without *akt-1(mg144)*, *pdk-1(mg142)*, *mec-3p::pdk-1(mg142), mec-17p::unc-112::gfp* and *mec-17p::pat-6::gfp*, or vibration. N>3 and p<0.0005 for all other strains compared to *ins-10(i)* alone.

Stress conditions modulate touch sensitivity through INS-10

An *ins-10p::gfp* fusion was expressed strongly in two pharyngeal neurons: the M4 motor neuron and the I5 interneuron (Figure 22a), and weakly and sporadically in the MC pharyngeal interneurons, RIS interneurons, and an additional pair of nerve ring interneurons tentatively identified as either the RMF cells or RMH cells. Expression occurred throughout larval development and adulthood. Single molecule fluorescent in situ hybridization (smFISH) confirmed *ins-10* transcripts primarily in two pharyngeal cells in the positions of M4 and I5 (Figure 20b). Laser ablation of the M4 and I5 neurons in late L4 stage animals resulted in adults that were partially insensitive to anterior touch within 24 hrs of ablation (Figure 22b). Ablating the M4 neuron prevents feeding, so the ablated animals were compared with mock ablated animals that were starved for 24 hrs. These results suggest that INS-10 secreted from the M4 and I5 cells modulates anterior touch sensitivity. Ablating I5 alone produced weak reduction of anterior touch sensitivity (from 4.3 (non-ablated) or 4.6 (mock-ablated) responses out of five touches to 3.6 responses out of five touches. $p=0.07$ compared to non-ablated animals and $p=0.03$ compared to mock-ablated animals), but ablating M4 alone produced no significant decrement in touch sensitivity. Because none of the *ins-10* expressing cells have synaptic connections to the main gentle touch circuit (Albertson and Thomson, 1976; Chalfie et al., 1985; White et al., 1986), INS-10 acts hormonally on the TRNs.

INS-10 expression was reduced by hypoxic conditions or by dauer formation. An *ins-10p* driven GFP is reduced by about eight and 20 folds in M4 and I5 neurons, respectively, in dauer larvae compared to starved L3 larvae (Figure 22c/e). Under the same conditions, a control RFP and/or GFP expressed in the pharyngeal muscles was

reduced by only three folds, and *mec-3* promoter driven RFP expressed in the ALM neurons was not reduced (Figure 22e). Similarly, in animals grown under hypoxic conditions (1% O_2 for 24 hrs), INS-10 expression in M4 and I5 neurons was reduced by four-fold and eight-fold, respectively (Figure 22d/f). In contrast, hypoxia reduced expression from *mec-3p::rfp* only two-fold, and caused no reduction of GFP expressed in the pharyngeal muscles (Figure 22f). These data suggest that the reduction of INS-10 expression was not solely caused by general reduction of translation during hypoxia response (Connolly et al., 2006) or generally reduced transcription in dauer larvae (Dalley and Golomb, 1992).

These treatments, as expected, also reduced anterior touch sensitivity. Animals grown under hypoxic conditions (Figure 23a) or in dauer larvae (Figure 23b), but not starved L3 animals were insensitive to anterior touch, supporting the hypothesis that INS-10 modulates anterior touch sensitivity under physiological conditions. In contrast, the anterior touch sensitivity of animals carrying an *akt-1* gain-of-function allele, *daf-16* loss-of-function allele, or animals expressing *pdk-1(mg142)* in the TRNs, were not affected by these conditions (Figure 23a/b). Animals carrying an *mfb-1* mutation, which restores the anterior touch sensitivity in *akt-1* animals, were also not affected by hypoxia. Therefore changes in anterior touch sensitivity under these conditions can be compensated by increased insulin signaling in the TRNs, consistent with the hypothesis that the changes in touch sensitivity was caused by *ins-10*. These results suggest that INS-10 may signal non-mechanical stress information to modulate TRN touch sensitivity through insulin signaling.

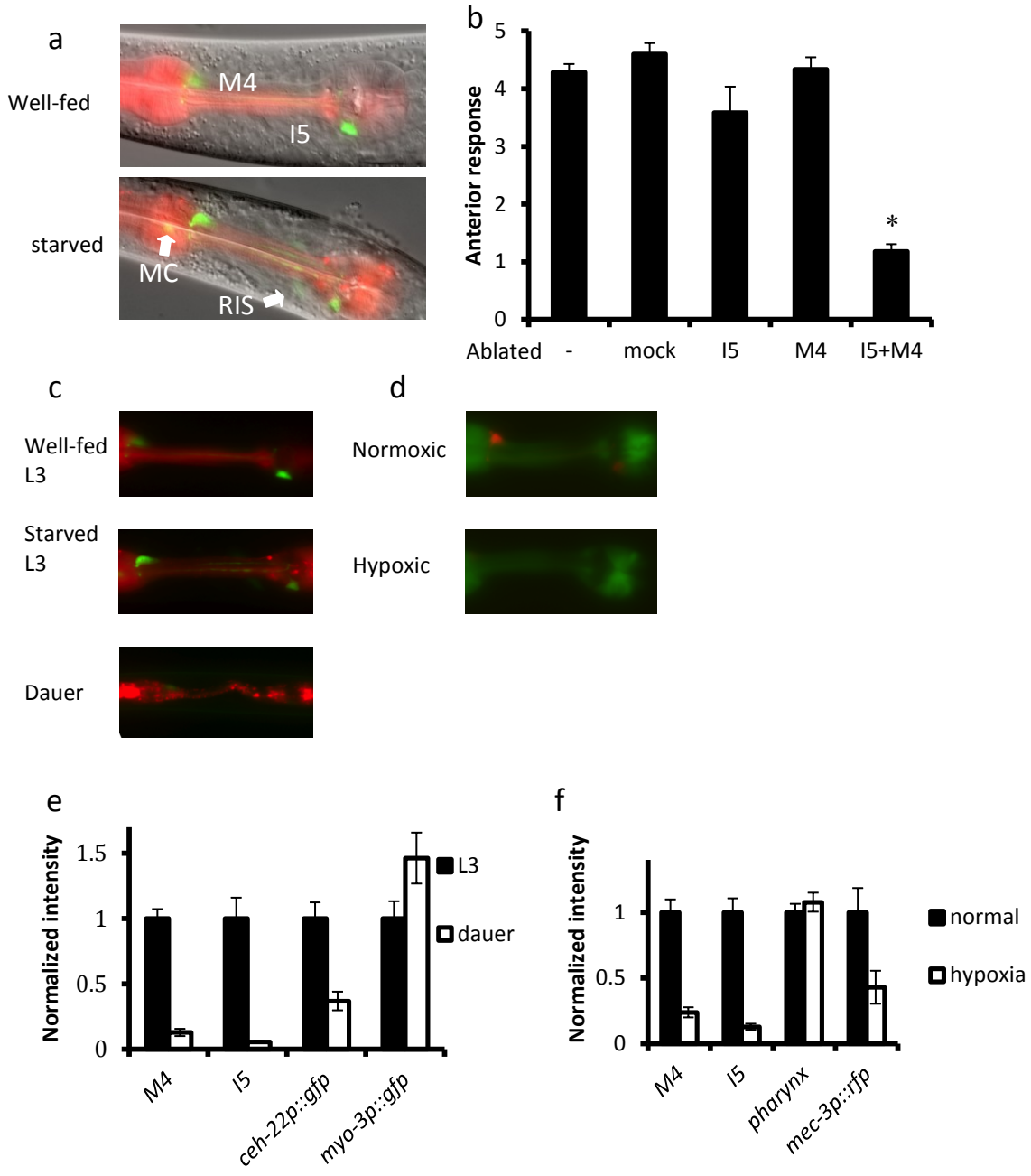

Figure 22. Regulation of *ins-10* expression under stress conditions. (a) *ins-10p::gfp* expression in well-fed and starved animals. The green channel showing GFP, the red channel showing *myo-2p::mcherry* and the DIC channels were merged. (b) Anterior touch response (mean ± SEM of individual animals) of animals with no ablation(-), mock ablation (mock), or with I5 and/or M4 ablated. N≥12 for all. *p<0.0001. (c)(d) expression

of *ins-10p::gfp* or *ins-10p::rfp* in well-fed L3 animals, starved L3 animals, dauer larvae (c), adult animals under normoxic or hypoxic conditions (d). *myo-2p::mcherry* and *ceh-22p::gfp* expression in the pharyngeal muscles were merged with the *ins-10* channels. (e)(f) The intensities (mean ± SEM of individual animals) of *ins-10p::gfp* or *ins-10p::rfp* in the M4 and I5 neurons, *ceh-22p:gfp* or *myo-2p::mcherry* in the pharynx, and *mec-3p::rfp* in the TRNs in starved L3 or dauer larvaes (e), or in adult animals under normoxic or hypoxic conditions (f). N≥5 for all cells measured.

High salt modulates touch sensitivity and INS-22 expression

We have previously found that high salt reduces anterior touch sensitivity of wild-type animals. Growing wild-type animals for three hours in high salt was enough to reduce touch sensitivity in adult animals (the initial drop in sensitivity after 30 min is due to an acute response to hyper-osmolarity); similarly, transferring adult animals from high salt conditions to normal conditions for three hours restored their touch sensitivity (Figure 24a). Because essentially all TRN development occurs before the adult stage (Chalfie and Au, 1989), these results suggest that high-salt modulates sensory signaling and not development. The reduction in touch sensitivity required a minimum of 100 mM NaCl, and reached the maximum at 230 mM NaCl. These changes were not a response to increased osmolarity, since they were not seen in animals raised in standard medium with 50 mM NaCl supplemented with 380 mM sucrose, which creates higher osmolarity than 230 mM NaCl (Figure 24b).

High salt, however, did not reduce the anterior touch sensitivity in animals carrying *daf-16(mgDf50)* loss-of-function mutation, *akt-1(mg144)* gain-of-function mutation, *pdk-1(mg142)* gain-of-function mutation or *mfb-1(gk311)* loss-of-function mutation (Figure 24c). Over-expressing *unc-112* and *pat-6*, or a construct containing a gain-of-function *pdk-1* equivalent to *pdk-1(mg142)* in the TRNs also restored anterior

touch sensitivity under high salt. These data indicate that increasing insulin signaling or integrin signaling in the TRNs compensates for the effect of high salt on anterior touch sensitivity.

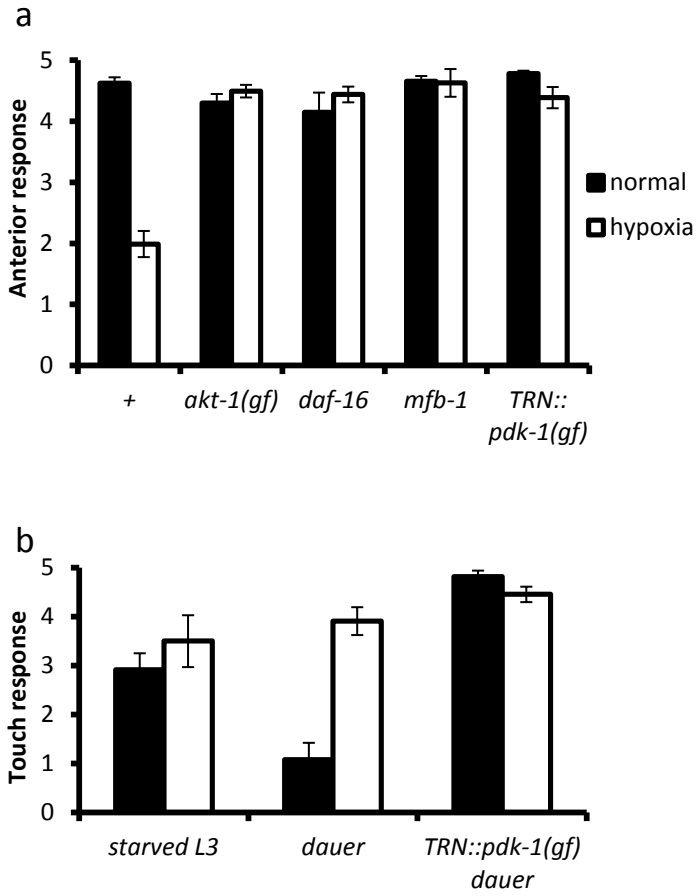

Figure 23. Anterior touch sensitivity of animals under stress conditions. (a) anterior touch sensitivity (mean ± SEM) of wild-type, *daf-16(mgDf50)*, *mfb-1(gk311)* or *akt-1(mg144)* animals, or animals carrying *mec-3p::pdk-1(mg142)* grown under normoxic or hypoxic conditions. N≥4 for all strains and conditions tested. P<0.0001 comparing wild type under hypoxia to other strains under hypoxia. (b) Anterior (black) and posterior (white) touch sensitivity (mean ± SEM of individual animals) of wild-type starved L3 or dauer larvaes with or without *mec-3p::pdk-1(gf)*. N>10 for all strains and conditions tested. P<0.001 comparing the anterior response of wild-type dauers to wild-type starved L3 larvae or dauer larvae expressing *mec-3p::pdk-1(gf)*.

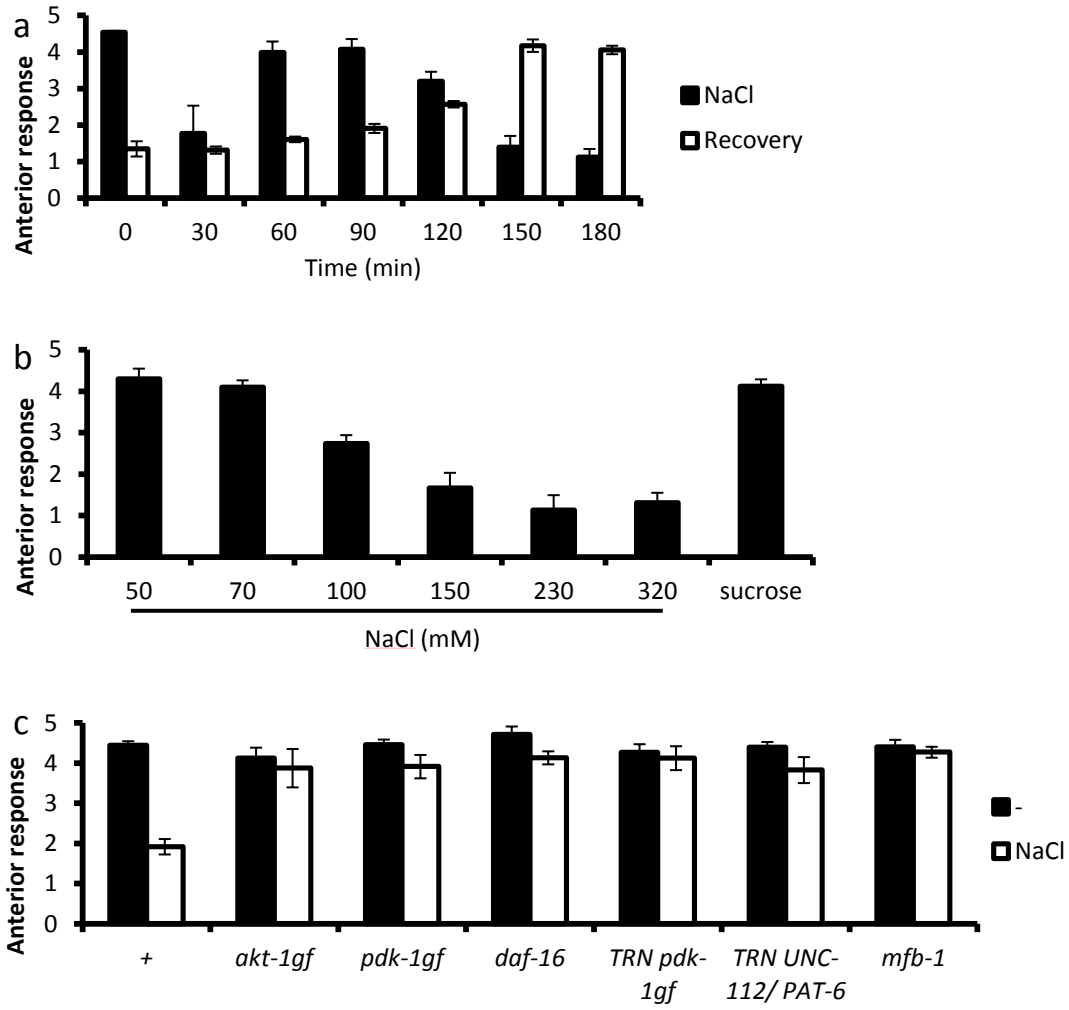

Figure 24. High salt modulates touch sensitivity. (a) Anterior touch response (mean ± SEM) of wild-type animals at the noted time points after they were transferred from 50 mM NaCl to 230 mM NaCl (NaCl) or from 230 mM NaCl to 50 mM NaCl (Recovery). N≥3 for all time points. (b) Anterior touch response (mean ± SEM) of wild-type animals grown on NGM plates with the specified concentration of NaCl, or with 50 mM NaCl and 380 mM sucrose. (c) Anterior touch response (mean ± SEM) of wild-type, *akt-1(mg144)*, *pdk-1(mg142)*, *daf-16(mgDf50)* or *mfb-1(gk311)* animals, or wild-type animals carrying *mec-3p::pdk-1(mg142)*, or *mec-17p::unc-112::gfp/mec-17p::pat-6::gfp* grown on NGM plates (50 mM NaCl) or with 230 mM total NaCl. N≥3 for all strains tested. $p<0.001$ comparing wild type grown on high salt to wild type grown on NGM plates or other animals grown on high salt.

The reduction of touch sensitivity under high salt is ion-selective. Na^+ or Cl^- alone was capable of inducing the reduction, although the effect was weaker than both together (Figure 25a). However, the same concentration of ammonium acetate did not reduce touch sensitivity (Figure 25a). This pattern of salt selectivity resembled the gustatory responses of ASE cells, which respond to Na^+ and Cl^- but not ammonium acetate (Ortiz et al., 2009). *che-1* animals that lack ASE cells were partially insensitive to anterior touch (Figure 25b), suggesting that the ASE cells may be responsible for modulating touch sensitivity in high salt. Similar to high salt induced touch insensitivity, the touch insensitivity of *che-1* mutant animals can also be restored by expressing gain-of-function *pdk-1* in the TRNs (Figure 25b), supporting the hypothesis that ASE cells sense high salt to modulate touch sensitivity.

ins-22 was expressed strongly in both ASE cells. Its expression was reduced when the animals were grown in high salt (Figure 25c), suggesting that high salt may partially modulate the touch response through *ins-22*. However, *ins-22* ALM neurons had normal force sensitivity as measured by calcium imaging (data not shown), suggesting that INS-22 does not act through the same insulin signaling in the TRNs as INS-10. High salt, therefore, probably modulates touch sensitivity in the TRNs through another pathway, perhaps through other unidentified insulin-like peptides.

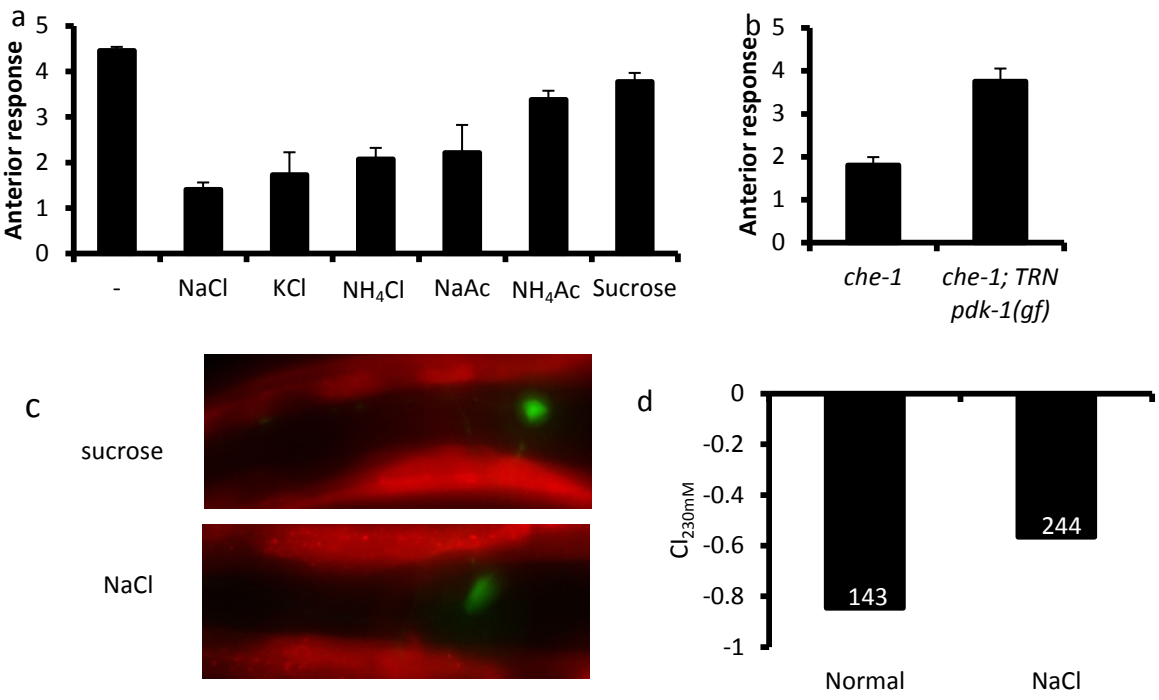

Figure 25. High salt modulate touch sensitivity through the ASE cells. (a) Anterior touch response (mean ± SEM) of wild-type animals grown on NGM plates supplemented with 180 mM of various salts, or 380 mM of sucrose. N≥3 for all conditions tested. $p<0.0001$ between NaCl and NH_4Ac or sucrose, $p<0.006$ between KCl, NH_4Cl and control, and $p<0.03$ between NaAc and control. (b) Anterior touch response (mean ± SEM) of *che-1* animals with or without *mec-3p::pdk-1(mg142)*. N≥3 for both strains. $p<0.002$. (c) *ins-22p::gfp* expression in animals grown on NGM plates (50 mM NaCl) supplemented with either 380 mM sucrose or 180 mM NaCl, overlaid with RFP expression in the body-wall muscle. (d) The chemotaxis index towards 230 mM NaCl over 130 mM NaCl in animals grown on NGM plates with 50 mM NaCl (normal) or 230 mM NaCl (NaCl). The total numbers of animals were combined from three independent experiments and are labeled on top. $p<0.0001$ against the null hypothesis of equal distribution (CI=0), and $p<0.0005$ comparing the distribution between animals grown on NGM plates and animals grown on high-salt plates.

Although NaCl is attractive to animals, a high concentration of NaCl is repulsive to animals, because the animals still move toward lower concentration of NaCl when

presented with a linear gradient from 130 mM NaCl to 230 mM NaCl regardless of whether the animals were grown at 50 mM NaCl or 230 mM NaCl before the test (Figure 25d). Animals grown at 230mM NaCl did show weaker preference to low salt, consistent with reports of gustatory associative learning (Saeki et al., 2001). Therefore, high salt is also a non-preferred condition that reduces touch sensitivity.

Modulation of touch sensitivity enhances task completion under mechanical distractions

The *ins-10* RNAi animals respond differently to mechanical distractions, such as non-localized vibration, from wild-type animals. To quantitatively assess responses to non-localized mechanical stimuli, we videotaped freely moving L4 or young adult animals under short pulses of vibration, and quantified the type of movement (forward/backward) and the turning angle of the animal after the response (See Experimental Procedures for details). Because vibration activates both anterior and posterior TRNs, the response to the vibration is determined by the balance between the anterior and posterior touch responses. Less *ins-10* RNAi animals moved backward than wild-type animals (Figure 26a; $p<0.0001$), consistent with our observation that the anterior touch response was weaker in the *ins-10* RNAi animals than in wild-type animals. Because backward movements induced by plate tapping are usually accompanied by changes in movement direction (Chiba and Rankin, 1990), *ins-10* RNAi animals changed directions of movement less frequently than wild type (Figure 26b). Therefore, the behaviors of *ins-10* RNAi animals are less affected by mechanical distraction than that of wild-type animals.

Such increased resistance to mechanical distraction may facilitate the completion of non-mechanical tasks. We tested how fast animals move to an attractant source (diacetyl) when the plates were tapped once every 30 sec (See Experimental procedures for details). Fewer wild-type animals reached the diacetyl side when the plate was tapped than without tapping (Figure 26c/d), suggesting that mechanosensory response interferes with the efficiency of chemotaxis. *ins-10* RNAi treated animals, however, were slightly more efficient at chemotaxis when tapped. Mechanical stimuli caused the differences in these experiments, because *ins-10* animals had normal chemotaxis (data not shown). Expression of *pdk-1(gf)* in the TRNs, which rescued the touch sensitivity of *ins-10* RNAi animals (Figure 21c), reduced the chemotaxis efficiency of *ins-10* RNAi animals with tapping (Figure 26c/d), suggesting that reductions of touch sensitivity in the TRNs was responsible for the change in chemotaxis efficiency under vibration. Therefore, reduction of anterior touch sensitivity increases an animal's efficiency at non-mechanosensory tasks under mechanical distractions. The increased efficiency may facilitate survival under stress conditions.

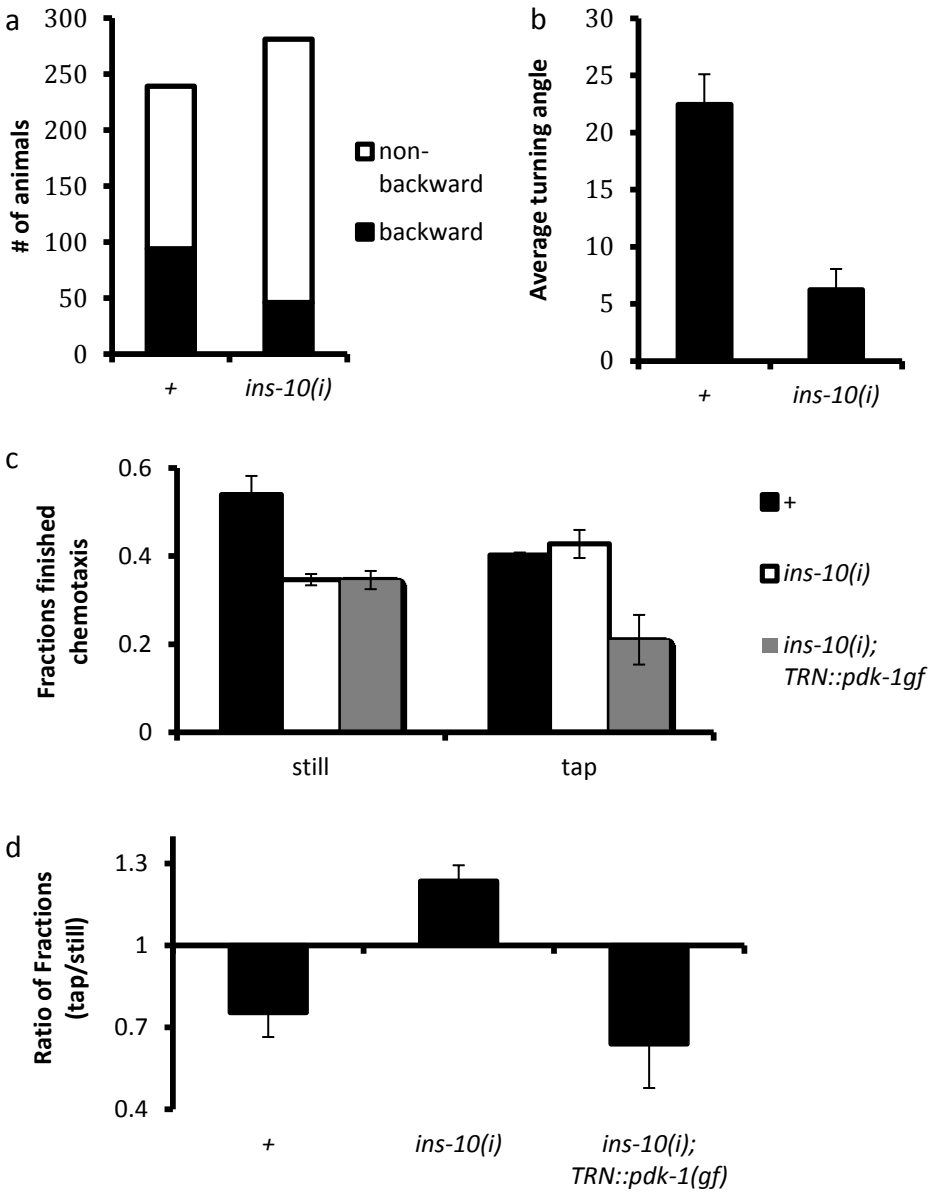

Figure 26. Reduced touch sensitivity increases chemotaxis efficiency. (a) The number of animals responding to a pulse vibration by backward movement or non-backward movement (forward or no movement) in wild-type and *ins-10(i)* animals. The numbers are pooled from four independent trials. $p<0.0001$. (b)The average turning angle (mean ± SEM of independent trials) of wild-type *and ins-10(i)* animals responding to pulse vibration. N=4 for each strain, $p<0.005$. (c) The fractions (mean ± SEM of independent trials) of wild type, *ins-10(i)* animals, or *ins-10(i)* animals expressing *mec-3p::pdk-1(mg142)* that have reached the diacetyl spot after 12 mins in a chemotaxis assay with or without tapping. $p<0.02$ for wild-

type animals with or without tapping. N≥3 for all strains. (d) The ratio of fractions of animals that have reached the diacetyl spot with or without tapping. $p<0.02$ between *ins-10(i)* and *ins-10(i); mec-3p::pdk-1(mg142)* and $p<0.01$ between *ins-10(i)* and wild type. N≥3 for all strains.

Discussion

Stress conditions de-prioritize mechanosensation through INS-10 signaling

Gentle touch along the body induces a fast escape response away from the stimulus. The speed of the response, determined both by the low latency of the MEC-4/MEC-10 channel (<0.5msec; O'Hagan et al., 2005) and the gap junction dominated circuitry (Chalfie et al., 1985), is crucial for successful escape from threats associated with mechanical stimuli (Maguire et al., 2011). The gap junctions connecting the TRNs with downstream command interneurons allow faster transmission, but less modulation than chemical synapses, and therefore are usually seen in circuits involved in escape responses (Furshpan and Potter, 1957; Waziri, 1969; Gillette and Pomeranz, 1975). In contrast, other sensory circuits that do not mediate an immediate escape response, such as chemotaxis circuits, are connected through a network of interneurons mostly through chemical synapses (Gray et al., 2005). Therefore, these escape responses, including the gentle touch response, have very high priorities over other sensory inputs in regulating the movement of the animal. Our results, however, suggest that the anterior touch sensitivity can be down-regulated under stress conditions.

It may be counterintuitive to weaken an escape response under stress conditions, but our result that animals with reduced INS-10 became more efficient at chemotaxis under vibration suggests a possible advantage for the animal. A strong anterior touch response leading to a change in the direction of the movement would disrupt the movement of an animal when it is trying to move away from stress conditions to favorable conditions (e.g. from hypoxic to normaxic conditions, or towards food when dauer-arrested), reducing its chance to survive. Therefore, reduced anterior touch

responses, complemented with possible enhancement in other sensory modalities such as chemotaxis (Pocock and Hobert, 2010), increases the chances of survival under stress conditions.

In addition, stress conditions do not completely eliminate touch sensitivity. The residual touch sensitivity is still capable of mediating an escape response from a direct touch with a heavier force. INS-10 mediated modulation thus maintains a balance between the sensitivity to mechanical force and the efficiency of the animal in non-mechanical tasks. We propose that down-regulation of touch sensitivity is needed to de-prioritize touch response so that the directional movement of the animal can be dominated by other senses, facilitating survival under stress conditions.

Insulin-like peptides modulate touch sensitivity

We have discovered multiple insulin-like peptides that affect touch sensitivity, including INS-10 and INS-22. INS-10 acts upstream of canonical insulin signaling in the TRNs to modulate touch sensitivity. Because integrin signaling and insulin signaling converge on AKT-1, reduction of either pathway should induce similar changes in touch sensitivity. Indeed, calcium imaging showed that INS-10 modulates force sensitivity, but not calcium signaling in the TRNs, similar to UNC-112.

INS-10 is expressed in the M4 pharyngeal motor neuron and I5 pharyngeal interneuron. The M4 motor neuron innervates the posterior isthmus of the pharynx and is essential for feeding (Avery and Horvitz, 1987). The I5 interneuron plays a minor role in feeding (Avery, 1993), but it also has desmosome attachments to nearby cells that may serve mechanosensory functions (Albertson and Thomson, 1976). These two neurons are connected with each other and with other pharyngeal neurons, but not with either the

TRNs or the downstream command interneurons (AVA, AVB, AVD and PVC). The lack of synaptic connection with the gentle touch circuitry supports the hypothesis that the M4 and I5 pharyngeal neurons modulates touch sensitive by secreting INS-10 as a long-distance neuromodulator. In support of this hypothesis, the M4 neuron expresses multiple neuropeptide genes, including *flp-2*, *flp-5*, and *flp-21*, in addition to *ins-10*. At least FLP-21 is secreted to affect social feeding behavior (Rogers et al., 2003) and gustatory circuitry (Pocock and Hobert, 2010) by acting on remote targets. Furthermore, as others (Pocock and Hobert, 2010) and we have shown, the expression of neuropeptides in these two cells can be modulated by various conditions, many of which are unlikely to be sensed by these two neurons directly. Therefore, despite their isolations from the main neuronal network, the M4 and I5 neurons may act as neuroendocrine hubs to modulate neuronal activities through long-range neuropeptides.

INS-22 also affects the touch response. Our data, however, suggest that INS-22 does not act on insulin signaling in the TRNs as INS-10 does, because *ins-22* mutant animals did not show reduced force sensitivity as measured by calcium imaging. INS-22 is expressed in the ASE cells, which synapse onto multiple interneurons that affect turning, including the AIA, AIB, AIY and AIZ cells. The ASE cells have been shown to signal to these downstream synaptic partners through neuropeptides (Chalasani et al., 2010). Therefore, it is possible that INS-22 acts directly on these interneurons instead of on distant neurons, and modulates turning rate in general. Such a circuit-level modulation would affect the touch response as measured by the touch assay, without affecting TRN force sensitivity.

Convergent modulation of mechanosensation through AKT-1

Sensory perception adapts to diverse contextual information. Previously, we have shown that the touch sensitivity can be modulated by sustained vibration through integrin signaling and AKT-1. AKT-1 regulates the amount of MEC-4 channel on the plasma membrane through MFB-1 dependent ubiquitination. In this paper, we have shown that touch sensitivity can also be modulated by non-mechanical contexts such as dauer formation and oxygen level through the insulin-like peptide INS-10. Paralleled to integrin signaling, INS-10 activates insulin signaling in the TRNs to activate AKT-1. Integrin signaling and insulin signaling compensate for each other, suggesting that their effects on AKT-1 are additive. These results suggest AKT-1 integrates both non-mechanical and mechanical contextual information through two different upstream signaling pathways.

High salt is sensed by the ASE cells and reduces touch sensitivity through an unknown pathway. This effect, however, can also be compensated either by increasing insulin signaling through gain-of-function mutations, or by increasing integrin signaling by vibration or over-expressing UNC-112 and PAT-6 in the TRNs, or by eliminating *mfb-1* through a loss-of-function mutation. These data suggest that high salt may also modulate touch sensitivity through insulin signaling and MFB-1, possibly by other insulin-like peptides. Our failure to identify such insulin-like peptides suggests that such insulin-like peptides may be redundant, so that knocking out a single insulin-like peptide produces only a mild effect on touch sensitivity.

Additional signaling pathways may also modulate touch sensitivity. Although we screened all insulin-like peptide genes, we may have missed insulin-like peptides that

inhibit insulin signaling and/or are not expressed under normal growing conditions, since the lack of such peptides would not reduce touch sensitivity. These inhibitory peptides, however, are unlikely to be involved in the effect of high salt on mechanosensation, because animals without the ASE cells were also touch insensitive, and therefore any signal from the ASE cells should positively affect touch sensitivity. In addition, other signaling pathways may also modulate touch sensitivity through cross-talk with insulin signaling. For example, we previously found that the RAS/MAPK pathway is needed for optimal touch sensitivity, and considerable cross-talk exists between the RAS/MAPK pathway and the PI3K/AKT pathway (Aksamitiene et al., 2012; Nakdimon et al., 2012; Nanji et al., 2005). Therefore, our findings that AKT-1 integrates multiple contextual signals, including mechanical signals through integrin signaling, non-mechanical stress signals through INS-10, and possibly high salt stress signals through unidentified insulin-like peptides, suggest that AKT-1 is a master modulator that incorporates multiple contextual signals into the modulation of mechanosensation.

Experimental procedures

Strains

C. elegans strains were maintained at 20°C, or 15°C for temperature-sensitive mutants as described by Brenner (1974) (see appendix II for a list of strains used).

Constructs

The feeding RNAi constructs for insulin-like peptides were made by cloning 500-1000 bp of the coding regions from cDNAs and inserted into L4440 through an EcorV site. The *ins-36* feeding RNAi construct and the *ins-10* coding region with alternative codons were synthesized by Genewiz, Inc. (South Plainfield, NJ) in L4440 and pUC57, respectively. The *ins-10* coding region was then cloned into pDONR221 using the Gateway system (Life technologies, Grand Island, NY). All other constructs were made using the three-fragment Gateway system (Life technologies, Grand Island, NY) per manufacturer's instructions (See appendix II for primers).

Feeding RNAi

Feeding RNAi experiments were performed as described by Calixto et al. (2010a).

Behavioral assays

We tested touch sensitivity as described in Chapter III. The mean and SEM of the means from multiple independent tests were calculated and reported unless noted.

The vibration response assays were performed as described in Chapter III using 50Hz 0.5 sec square pulses with a maximum plate acceleration of 1.7±0.1g. Each plate was tested no more than twice with at least ten minutes in between.

The chemotaxis assay with tapping was performed using a mechanical tapper driven by a magnetic relay, as described by Rankin et al. (1990). The relay was controlled by a programmable power supply that is turned on for one second for every thirty seconds. 1 µl of 1% diluted diacetyl and 1 µl 1 M sodium azide was spotted 10 mm away from the side of a 10 cm test plate. A circle with a diameter of 20 mm was then drawn around the spot as the destination area. Adult animals were washed twice with M9, and starved for 15 minutes on an empty plate before being stripped to the test plate on the side opposite to the diacetyl spot with an inoculation loop, approximately 10 mm from the side of the plate. The strip of agar was then immediately removed from the test plate and the plate was taped and put on the tapper for 12 mins. After 12 mins, animals inside and outside of the 20mm circle was counted, respectively, to calculate the percentage of animals that have reached the diacetyl spot.

Dauer inductions and hypoxic growth conditions

Dauers were obtained by growing mixed-stage animals at 25°C for one generation and further starving for five days. The hypoxic treatment was performed as described by Pocock and Hobert (2010).

Laser ablations

Laser ablations were performed as described by Tsalik and Hobert (2003). The M4 and I5 cells were labeled with GFP and ablated in L4 larvae. All animals were anesthetized with 30 mM sodium azide for ablation. Control animals were left in sodium azide on a slide with the same duration as ablated animals, and mock ablated animals had adjacent pharyngeal neurons ablated instead of the M4 and I5 cells. After 24 hrs, the ablation was confirmed by the lack of GFP recovery in the M4 and I5 cells. The confirmed animals were then blinded and tested for touch sensitivity.

Microscopy

All images were taken on a Zeiss Observer Z1 microscope with a Photometrics CoolSnap HQ2 camera (Photometrics, Tucson, AZ). Quantifications of intensities were performed using ImageJ (Schneider et al., 2012). The single-molecule mRNA FISH was performed as described by Topalidou et al. (2011). Calcium imaging was performed as described in Chapter III.

Chapter V. Conclusions and future directions

In this thesis research, I used neuronal-enhanced feeding RNAi to identify pleiotropic genes affecting mechanosensation in the TRNs and found two converging pathways modulating touch sensitivity. The first pathway, integrin signaling, mediates mechanosensory modulation by the mechanical environment in a cell-autonomous fashion. The second pathway, long-range insulin signaling, mediates mechanosensory modulation by non-mechanical factors sensed by non-TRN cells. Both pathways signal through AKT-1, DAF-16 and MFB-1, and modulate touch sensitivity by regulating the amount of MEC-4 channels on the surface of the plasma membrane (Figure 27). These findings provide insights into how mechanosensensory modulation integrates information from multiple senses, and how these signals are coordinated to facilitate survival under diverse conditions.

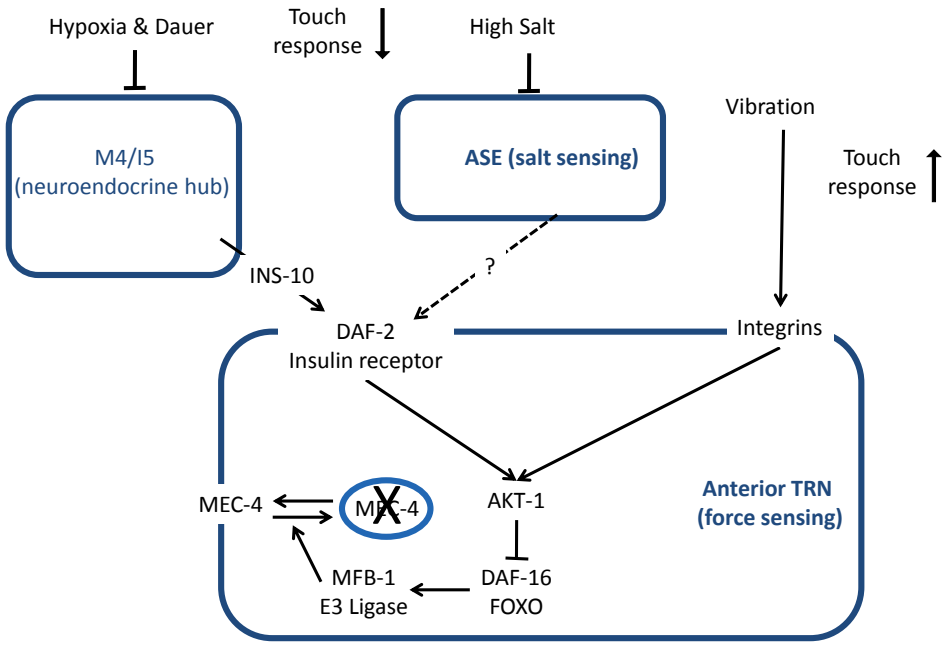

Figure 27. Modulation of mechanosensation. The sensitivity to force in the TRNs is modulated by stress conditions or external force through insulin or integrin signaling. Both signaling pathways converge on AKT-1 and change the force sensitivity by controlling the amount of MEC-4 mechanotransduction channel through ubiquitination.

Strategies for intra- and inter-modal mechanosensory modulation

In *C. elegans*, mechanosensation in the TRNs can be modulated both by signals sensed by the same sensory cells (intra-modal modulation), such as previous experience of touch stimuli (Rankin et al., 1990), and by signals sensed by other sensory cells (inter-modal modulation), such as the texture of a bacteria lawn (Kindt et al., 2007). I have extended the understanding of mechanosensory modulation by describing two additional pathways that facilitate intra- and inter-modal modulation, respectively. Integrin signaling increases touch sensitivity when the animals are under background vibration. Insulin signaling decreases touch sensitivity under several non-mechanical stress conditions. These two pathways provide an example of how an animal feeds information from multiple senses into mechanosensory neurons.

1. Intra-modal modulation by a second mechanosensory system

Mechanical signals modulate touch sensitivity in the TRNs utilizing integrin signaling in a cell-autonomous fashion. Although MEC-4 channels are responsible for neuronal mechanotransduction in the TRNs, the MEC-4 channels themselves and/or neuronal activities are not needed for mechanosensory modulation by mechanical force. Because the integrins are mechanically sensitive (Chen, 2008; Roca-Cusachs et al., 2009; Vogel and Sheetz, 2009), we speculate that the integrins act directly as mechanosensors in TRN modulation by force.

Using two different mechanosensors in the same cells for mechanosensation and its modulation provide flexibility to the system. The TRNs are positioned close to the surface of the animal and attached to the hypodermis, and therefore are physically

suitable for sensing gentle touch along the body. The MEC-4 channels sense changes in pressure applied at a certain point on the body, but these sensors do not sense constant force, and therefore do not provide an accurate description of the mechanical environment of the TRNs. The steep force-response curves measured by both electrophysiology (O'Hagan et al., 2005) and calcium imaging also suggests that the MEC-4 sensor only responds differentially to force in a very small range, adding to the one-dimensionality of the system. These deficiencies, however, are acceptable because the main function of the TRNs is to generate an escape response, so being able to detect forces promptly is more important than having an accurate measurement of the mechanical environment. To compensate for the lack of information, the integrins sense constant forces generated by stretching and pulling (Chen, 2008; Vogel and Sheetz, 2009), and modulate the MEC-4 channels accordingly. In contrast to the MEC-4 channels, integrins sense forces slowly because it signals through a signaling cascade involving AKT-1 and transcriptional regulation of MFB-1, possibly by DAF-16. In the example that I have shown, the increased sensitivity of MEC-4 after sustained vibration counters the gain reduction by habituation, thus maintaining a functional gentle touch system under sustained background vibration. Therefore, by complementing the fast-acting MEC-4 channels with the slower-acting integrins in the TRNs, the MEC-4 channels can be adapted to a variety of mechanical environments to optimize detection without losing its low latency characteristics.

2. Inter-modal modulation through long-range neuromodulation

Signals sensed by other sensory cells modulate mechanosensation through a cell non-autonomous mechanism. Our findings of insulin-mediated modulation, and the

previous reports of long-range dopamine modulation by Kindt et al. (2007), suggest that mechanosensation in the TRNs is affected by long-range neuromodulation. Similar long-range neuromodulation has previously been observed in both mechanosensation (Kindt et al., 2007) and chemosensation (Pocock and Hobert, 2010). This feature may be partially due to the fact that the TRNs, like other sensory neurons, receive relatively little synaptic input. If modulation across different sensory circuits rely on synaptic modulation, a large number of interneurons would need to be committed to relaying modulatory signals across these sensory circuits. In addition, each modulatory factor may change a distinct subset of senses, suggesting that each interneuron that relays modulatory signals across senses would have to be connected with distinct sensory neurons, further complicating the circuit. In the simple *C. elegans* nervous system of 302 neurons, such a scheme would be impossible given the limited number of neurons that are pre-synaptic to the TRNs and possibly other sensory neurons. Long-range neuromodulation, however, simplifies the circuit considerably. By expressing different combinations of neuropeptides and receptors in different sensory neurons, such a system enables diverse neurons, sensing different modulatory factors, to modulate distinct sets of target neurons.

The *C. elegans* genome contains 40 insulin-like genes, 34 *flp* genes, and 42 *nlp* genes that encode neuropeptides. Many *flp* and *nlp* genes encode multiple neuropeptides per gene. The expression of these neuropeptides, however, seem to be concentrated in some neurons. The M4 pharyngeal neuron, for example, expresses *flp-2*, *flp-5* and *flp-21* in addition to *ins-10* (Li and Kim, 2008; Pocock and Hobert, 2010). The ASE neurons express *ins-1*, *flp-6*, *flp-13*, *nlp-3* and *nlp-7* (Li and Kim, 2008) in addition to *ins-22* (Etchberger et al., 2007). In comparison, the ALM and AVM neurons are only known to

express *flp-20* (Li and Kim, 2008). Although not all neuropeptides are secreted hormonally, such patterns of expression suggest that some neurons may act as neuro-endocrine hubs that integrate information and modulate multiple neurons through different neuropeptides. These hubs would therefore act like the interneurons in a synaptic neuronal circuit, integrating information from multiple inputs and signaling to multiple downstream targets. Such a model would explain how the expression of *ins-10* in the M4 and I5 pharyngeal interneurons is modulated by many seemingly unrelated stress conditions, despite the fact that these two neurons are isolated from the main circuit and presumably function mainly in the pharynx. Understanding the organization of such long-range neuromodulatory "circuits" may be as important as understanding the synaptic connectome of a nervous system.

Integration of modulation by multiple senses through a conserved common mechanism

Although intra-modal modulation through the integrins and inter-modal modulation through INS-10 initiate from different cells, both pathways converge in the TRNs at AKT-1 and DAF-16, and modulate touch sensitivity through a common mechanism. Because integrin signaling and insulin signaling compensates for each other in mechanosensory modulation, their effects through AKT-1 appear additive. Such convergence suggests that AKT-1 and DAF-16 integrate modulation by multiple factors and senses.

Additional insulin-like peptides may also modulate touch sensitivity. Although we have tested the effects of mutations or feeding RNAi treatments against genes encoding all insulin-like peptides, we would have missed insulin peptides that antagonize

insulin signaling, such as INS-1 and INS-18 (Pierce et al., 2001; Matsunaga et al., 2012), and agonistic insulin-like peptides that are expressed only under specific conditions, not under normal conditions. Therefore, the modulatory network of touch sensitivity by insulin signaling may be much more complicated than what I have described here.

Insulin signaling may cross-talk with additional signaling pathways. I have identified several other signaling pathways, including the Ras/MAP kinase pathway and the Rho GTPases, as needed for touch optimal sensitivity. Both Ras and Rho GTPases contribute to PI3K activation in diverse processes, such as cell adhesion, proliferation and migration (Radeff-Huang et al., 2004; Sasaki and Firtel, 2006; Andrews et al., 2007). Cross-talk between the LET-60 Ras GTPase and insulin signaling in *C. elegans* is involved in modulating dauer formation and longevity (Nanji et al., 2005), and cross-talk between the UNC-73 Rho GEF, LET-502 Rho-binding kinase, and insulin signaling is involved in embryonic morphogenesis (Piekny et al., 2000). In the TRNs, *unc-73* is needed for ALM cell migration and axonal guidance (Hedgecock et al., 1987; Siddiqui, 1990; Steven et al., 1998). Although these defects were not present in insulin signaling mutants, the Rho GTPases may play additional roles in modulating touch sensitivity through insulin signaling, which could have been masked in *unc-73* mutants because of developmental defects. Further characterization of these additional pathways is needed to elucidate the full signaling map for mechanosensory modulation.

AKT-1 modulates touch sensitivity through ubiquitination-dependent regulation of MEC-4 trafficking. Loss of *akt-1* reduces the amount of MEC-4 on the plasma membrane, causing touch insensitivity. The membrane trafficking of the mammalian ENaC channels is mediated by the E3 ubiquitin ligase Nedd4.2, but the C-terminal

domain of human ENaC channel subunits that binds to Nedd4.2 is missing in MEC-4 and MEC-10. No other Nedd4.2 recognition site (L/PPxY) is present in either MEC-4 or MEC-10. In contrast, the membrane trafficking of MEC-4 channels is controlled by MFB-1, an E3 ubiquitin ligase homologous to the mammalian atrogin-1. The mammalian atrogin-1 functions in muscle atrophy downstream of the AKT kinase in muscles, which is activated by muscle contraction or passive muscle stretch through integrin signaling (Sakamoto et al., 2003; Hornberger et al., 2004; Zou et al., 2011). AKT kinase represses a Foxo transcription factor, which directly activates the transcription of atrogin-1 (Sandri et al., 2004). In *C. elegans*, loss of *mfb-1* activity enhances the constitutive dauer phenotype caused by reduced TGF-β signaling (Aoyama et al., 2004). Our findings suggest a new function of MFB-1 in modulating the amount of surface MEC-4 channels. Because both the mammalian atrogin-1 and the *C. elegans* MTB-1 mediate cellular responses to mechanical stimuli through integrin signaling, AKT kinases and Foxo transcription factors, this signaling pathway represents a conserved modulatory response to the mechanical environment.

Survival advantages of mechanosensory modulation in a natural environment

In this thesis, I have shown that the AKT-1 mediated modulation of mechanosensation can either increase or decrease touch sensitivity under physiological conditions. The bi-directionality of mechanosensory modulation under physiological conditions suggests that touch sensitivity is maintained at an optimal level under specific conditions. The significance of such modulation, however, must be considered in a natural environment instead of in a laboratory setting. In a natural environment of *C. elegans*, such as compost heaps or decaying fruits (Barriere and Felix, 2006), the TRNs

receive diverse mechanical stimuli. Some of these stimuli are not associated with particular threats, and are therefore non-informative, such as vibration caused by nearby large animals, or protrusions on the surface the animal crawls on. However, other stimuli predict dangers, such as contacts with fungi (Maguire et al., 2011) or other predators. How to distinguish biologically-relevant stimuli from irrelevant stimuli, therefore, is crucial to the survival of the animal. Modulation of touch sensitivity optimizes the detection of biologically-relevant stimuli in a background of non-informative stimuli.

To distinguish non-informative background vibration from stronger touch stimuli, the TRNs are modulated by integrin signaling. Under sustained vibration, the touch sensitivity of TRNs is modulated by two counteracting effects: the touch response is reduced by habituation, and touch sensitivity is increased by integrin signaling. We could detect the sensitization caused by integrin signaling by letting the animals recover from habituation before touch tests, but under natural conditions with continued background stimuli, sensitization compensates for the effect of habituation. If no sensitization occurs, as in *akt-1* mutant animals, habituation further reduces touch sensitivity, leaving the animal unresponsive to light mechanical stimuli and defenseless against possible mechanical threats associated with such stimuli. Therefore, the combined effect of habituation and sensitization maintains a balance for TRN responses between over-reaction to non-informative stimuli and sensitivity to strong stimuli that are potentially associated with danger. This balance minimizes energy waste caused by unnecessary escape reflexes while maintaining the ability to escape from danger.

Insulin signaling mediates reduction of touch sensitivity under stress conditions. The reduced touch sensitivity is inherently not advantageous to the animal because it

reduces protection against mechanical stimuli. The touch response is normally prioritized over other responses in controlling an animal's behavior: touching a wild-type animal almost always induces an escape response, whereas other forms of sensory perception, such as chemotaxis, only increase or decrease the frequency of turning. In addition, gap junctions directly connect the TRNs to the command neurons, ensuring robust and fast responses. However, animals under stress conditions are constantly under threat from stress. Mechanical stimuli thus become a lesser threat in comparison, especially non-localized stimuli, which are not necessarily associated with danger. Under these conditions, insulin signaling de-prioritizes the touch response relative to other tasks to increase the efficiency in other tasks when non-informative background mechanical stimuli are present. The residual anterior touch sensitivity, however, is still sufficient to elicit a touch response if a stronger force is directly applied to the anterior of the animal, which may be an indicator of danger. Therefore, insulin signaling maintains the balance between the touch response and responses of other senses. Such balance maximizes the survival probability under stress conditions by trading touch sensitivity for efficiency in other tasks.

Topalidou and Chalfie (2011; unpublished data) have shown that two GABA receptor subunits, GAB-1 and LGC-37, are expressed in the TRNs, suggesting that the TRNs may be modulated by GABA. Several classes of GABA producing neurons, including the VD and DD motor neurons and the RME inter/motor neurons, are involved in proprioceptive circuits (Schuske et al., 2004; Jorgensen, 2005; Wen et al., 2012), and therefore may transmit proprioceptive information to the TRNs to coordinate motor functions and mechanosensation, similar to the GABA-mediated pre-synaptic inhibition

of proprioception in the crustacean afferent neurons by motor neurons. In addition, Kindt et al. (2007) have shown that the CEP neurons sense the texture of bacteria lawn and decrease the rate of habituation in the ALM neurons when an animal is on food by releasing dopamine. Therefore, similar to the insulin modulation, both of these mechanisms balance mechanosensory responses with other sensory or motor functions: in the absence of food (a stress condition), the ALM neurons habituate faster because of the lack of dopamine, thus reducing the effect of background mechanical distraction on the behavior of the animal; activation of the GABAergic neurons suppress TRN activation, reducing the chance of accidental activation during movement (Figure 28).

In summary, modulation of the TRNs achieves balance under diverse conditions: modulation by non-TRN neurons maintains the balance between mechanosensation and other sensory/motor functions, and modulation within the TRNs (habituation and integrin-mediated sensitization) balances sensitivity to biologically-relevant stimuli and over-reaction to background stimuli (Figure 28). These mechanisms maintain an optimal mechanosensory response while coordinating different sensory and motor functions to maximize efficiency of the animal.

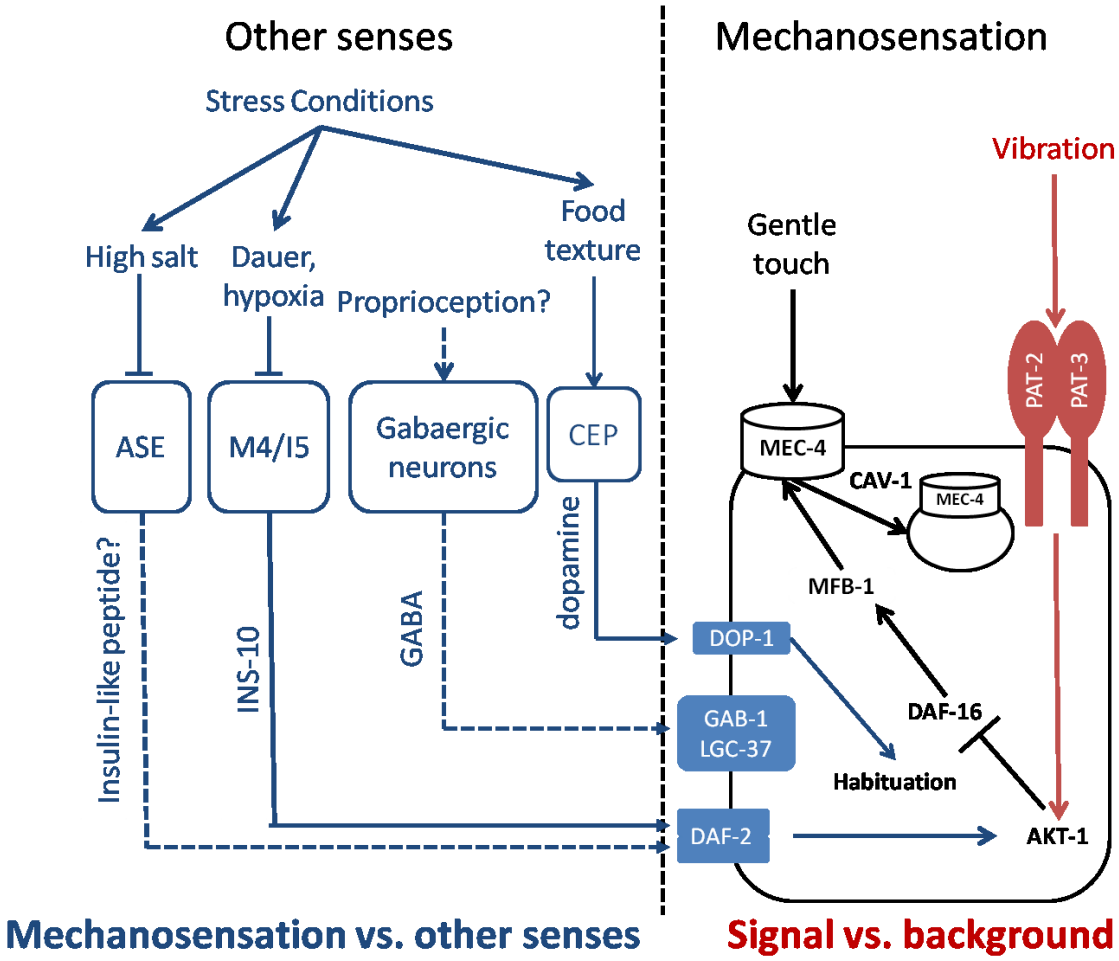

Figure 28. Model of integrated mechanosensory modulation. Mechanosensory modulation maintains the balance between sensitivity to biologically relevant stimuli vs. background stimuli (red) through integrin signaling and habituation, and the balance between mechanosensory response and other sensory responses through insulin peptide signaling, dopamine, and possibly GABA (blue). Both integrin and insulin pathways converge on AKT-1 and modulate the surface expression of MEC-4 through MFB-1 (black). Modulation maintains optimal touch response under diverse conditions and coordinates the touch response with other sensory/motor functions.

Future directions

I have described two converging pathways, insulin signaling and integrin signaling, that modulate touch sensitivity in the TRNs. Both pathways maintain a sensitive mechanosensory system under diverse conditions and minimize responses to non-informative stimuli. However, questions still exist as to the detailed mechanisms of the modulatory system, and how modulatory information is integrated to affect touch sensitivity.

As a stretch sensor, the integrins may detect other forces as well. For example, they may be activated by longitudinal tensions along the body of the animal. Higher stretching forces may decrease sensitivity to force, because the increased tension on the hypodermis would reduce displacement caused by a touch stimulus. Under such conditions, the increase in integrin signaling may compensate for the loss of sensitivity caused by the mechanical changes in the animal. If integrins indeed mediate such modulation, integrin signaling may mediate general force-induced mechanosensory modulation.

My results showed that AKT-1 acts downstream of the focal adhesion complex in integrin signaling. However, how the focal adhesion proteins activate AKT-1 is unclear. One hypothesis is that AKT-1 can be phosphorylated by PAT-4 integrin linked kinase (ILK), as in cultured mammalian cells (Persad et al., 2001). However, other evidence suggests that the integrin-linked kinase functions normally without its kinase activity (Wickstrom et al., 2010; Hannigan et al., 2011). For example, a kinase-dead version of *pat-4* is sufficient to rescue the Pat phenotype of *pat-4* mutants (Mackinnon et al., 2002; however, I have tried repeating this experiment, but could only rescue the Pat phenotype

of *pat-4* mutants with wild-type *pat-4*, not with the kinase-dead *pat-4*). It is possible that although the ILK can perform some functions without its kinase, other functions, such as mechanosensory modulation, require its kinase activity. Therefore, it would be important to test the role of PAT-4 in mechanosensation using mosaic analysis, and whether its kinase activity is needed by rescuing *pat-4* null animals with a kinase-dead *pat-4*. Further in vitro biochemical analysis of the PAT-4 kinase activity would then be able to determine whether AKT-1 is a substrate for PAT-4.

AKT-1 modulates touch sensitivity through DAF-16 and transcriptional regulation of MFB-1 expression. These results suggest a simple model in which DAF-16 regulates *mfb-1* transcription, supported by my experimental data, DAF-16 binding data from the ModEncode project (Celniker et al., 2009), Foxo binding consensus sequence, and similar regulations in mammalian cells. However, to prove that DAF-16 regulates *mfb-1* expression, it is still necessary to show DAF-16 activation of the *mfb-1* promoter using an in vitro assay. We can also mutagenize the DAF-16 binding sites in the *mfb-1* promoter to create a promoter GFP fusion to prove that DAF-16 indeed regulates *mfb-1* transcription in vivo.

AKT-1 is also activated by insulin signaling from INS-10. Although we have shown that INS-10 expression changes under several conditions, it is not known how these conditions modulate INS-10 expression from the M4/I5 pharyngeal neurons. Because of their isolated position in the neuronal circuit, neither M4 nor I5 is likely to receive direct synaptic inputs from non-pharyngeal sensory neurons. Although some environmental information may be sensed by the putative mechanosensory endings in I5, or by other pharyngeal neurons and transmitted to the M4 and I5 cells through synapses,

other information may be transmitted from non-pharyngeal neurons through extra-synaptic neuromodulators, as is the case for hypoxia-induced gustatory modulation (Pocock and Hobert, 2010). This suggests, as discussed in Chapter IV, that the M4 and I5 neurons may act as neuroendocrine hubs for long-range neuronal modulation. To determine how INS-10 expression is regulated, I have tried an EMS mutagenesis screen in a strain carrying *ins-10p::rfp* to identify mutations affecting *ins-10* transcription in the hope of identifying upstream genes needed for sensing environmental conditions, which could be expressed in the sensory cells. The screen, however, yielded limited number of mutants. The difficulty partially lies in the process of visually identifying animals with altered RFP expression, which may be facilitated using a worm sorter. In addition, some genes may modulate INS-10 translation or secretion instead of transcription. These genes would not have been found with our screen. Using an *ins-10* GFP translational fusion to repeat the above mutagenesis screen using worm sorter may help elucidating how INS-10 expression and secretion is controlled on a circuit and cellular level.

INS-10 may not be the only insulin-like peptide modulating touch sensitivity. Our data showed that multiple insulin-like peptides affect the touch response, although some of them may affect the downstream circuit rather than TRN mechanosensation. How these different insulin-like peptides maintain their specificities is unknown, especially since only one insulin receptor (DAF-2) has been identified. The specificity could partially be produced by physical sequestration of different insulin-like peptides. It is possible that an insulin-like peptide may only be able to travel a short and act locally, or act on distant targets if they are released into structures that may support endocrine signaling, such as the pseudocoelom. However, the ALM and AVM neurons are not

particularly exposed to the pseudocoelom, suggesting that exposure to the pseudocoelom is not a requirement for long-range neuromodulation. In addition, insulin-like peptides are differentially expressed on a temporal scale (Baugh et al., 2011). Alternatively, the vastly diverse secondary structures of insulin-like peptides (Pierce et al., 2001) suggest that specificities among insulin-like peptides may be determined by structural differences. Insulin-like peptides with different structures may have different effects on the insulin receptor DAF-2, or that they may bind different receptors. Although DAF-2 is the only identified insulin receptor homolog, non-insulin receptors, such as G-protein coupled receptors, are involved in the signaling of relaxins, a family of mammalian insulin-like peptides. Indeed, many G-protein coupled receptors are differentially expressed in the anterior and posterior TRNs (Zheng and Chalfie, unpublished data), suggesting that they may be involved in the differential regulation of anterior and posterior touch sensitivity. The specificities among different insulin-like peptides ensure that a specific environmental condition modulates a defined set of neurons. Identifying additional insulin-like peptides that either act redundantly or decrease touch sensitivity, or other neuropeptides such as those encoded by the *flp* and *nlp* genes, and subsequently determining the downstream receptors and pathways for each of these neuropeptides would elucidate how the insulin-like peptides maintain their specificities.

My results provide a model of how mechanosensory modulation from different sources are integrated in the TRNs through a common pathway. However, such a model of modulation integration may be incomplete. Although insulin signaling and integrin signaling converge on AKT-1, the molecular mechanism remains unknown. Do they activate AKT-1 by phosphorylation at the same site or different sites? If phosphorylation

occurs at different sites, do these different phosphorylations mediate additional interactions with DAF-16 independent downstream signaling? Additional biochemical and genetic studies with AKT-1 mutated at specific phosphorylation sites would help answer these questions. In addition, as discussed above, many G-protein coupled receptors are expressed in the TRNs, suggesting that additional neuropeptides may modulate touch sensitivity. Modulation by these neuropeptides could be additive, or one modulation could override the effect of another modulation. Understanding how these additional neuropeptides modulate touch sensitivity would be an important step in understanding how the TRNs integrate a wide range of information to modulate mechanosensation.

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Appendix I. Enhanced neuronal RNAi in *C. elegans* using SID-1

(The following paper, published in *Nature Methods* in June 2010, contains data described in Chapter II. My contribution to this paper is limited to Figure 5, which is equivalent to Figure 2 in this thesis)

Enhanced neuronal RNAi in *C. elegans* using SID-1

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We expressed SID-1, a transmembrane protein from *Caenorhabditis elegans* that is required for systemic RNA interference (RNAi), in *C. elegans* neurons. This expression increased the response of neurons to double-stranded (ds)RNA delivered by feeding. Mutations in the *lin-15b* and *lin-35* genes enhanced this effect. Worms expressing neuronal SID-1 showed RNAi phenotypes when fed with bacteria expressing dsRNA for known neuronal genes and for uncharacterized genes with no previously known neuronal phenotypes. Neuronal expression of *sid-1* decreased nonneuronal RNAi, suggesting that neurons expressing transgenic *sid-1(+)* served as a sink for dsRNA. This effect, or a *sid-1(−)* background, can be used to uncover neuronal defects for lethal genes. Expression of *sid-1(+)* from cell-specific promoters in *sid-1* mutants results in cell-specific feeding RNAi. We used these strains to identify a role for integrin signaling genes in mechanosensation.

Since its discovery[1], RNA interference (RNAi) has served as a powerful tool to study gene function, especially in *Caenorhabditis elegans*. *C. elegans* is unusual in that it exhibits systemic RNAi; double-stranded (ds)RNA in the environment can enter and spread throughout the worm to silence the targeted gene[2,3]. RNAi occurs when worms are soaked in solutions of dsRNA or fed bacteria expressing dsRNA (feeding RNAi), yielding a powerful tool for reverse genetics in this organism[4,5].

The transmembrane protein SID-1 is essential for systemic RNAi in *C. elegans* because it allows the passive cellular uptake of dsRNA[6]. *Drosophila melanogaster* have robust cell-autonomous RNAi but lack both systemic RNAi and a SID-1 homolog[7]. *Drosophila* cells[6,7] or mouse embryonic stem cells[8] expressing the *C. elegans* SID-1, however, respond to dsRNA in the medium. Feeding RNAi is robust in virtually all cells in *C. elegans* except neurons[5,9], but RNAi occurs in neurons when dsRNA is produced in the neurons themselves[10]. Thus, the lack of a neuronal response to systemic RNAi does not reflect an inability of these cells to execute RNAi but correlates with the pattern of detectable SID-1; SID-1 is present in all cells outside the nervous system, but very few cells within it[6,7].

Here we investigated whether the lack of detectable SID-1 renders neurons refractory to systemic dsRNA. Neurons expressing *sid-1* responded efficiently to feeding RNAi. This allowed us to produce strains that are hypersensitive to systemic neuronal RNAi. Moreover, specific expression of *sid-1* in worms otherwise missing the gene allowed us to generate strains that display cell-specific feeding RNAi.

RESULTS

Expression of *sid-1* in neurons enhances neuronal RNAi

We generated several strains with chromosomally integrated arrays of the wild-type *sid-1* gene expressed from the pan-neuronal *unc-119* promoter ($P_{unc\text{-}119}$*sid-1*) to test whether SID-1 can increase neuronal RNAi. Unless noted otherwise, we used one of these generated strains, TU3270. TU3270 also contained *yfp* expressed from the *unc-119* promoter ($P_{unc\text{-}119}$*yfp*), so that RNAi for *yfp* could be assessed in all neurons, as well as *mec-6* expressed from the *mec6* promoter ($P_{mec\text{-}6}$*mec-6*). We also generated additional transgenic strains carrying $P_{unc\text{-}119}$*sid-1* (Online Methods). One strain was similar to TU3270 but lacked the *mec-6* transgene (TU3311); the other strain (TU3356) also lacked the *mec-6* transgene but replaced $P_{unc\text{-}119}$*yfp* with a different marker array (Online Methods). As a control we generated strain TU3310 with only a $P_{unc\text{-}119}$*yfp* array. All three $P_{unc\text{-}119}$*sid-1*-expressing strains gave similar responses to feeding RNAi, did not have defects in neuronal morphology as judged by fluorescence intensity and did not have any obvious behavioral abnormalities.

We tested for enhanced RNAi in neurons by feeding bacteria expressing dsRNA for *gfp* to $P_{unc\text{-}119}$*sid-1* and control worms. (The YFP protein sequence differs from GFP by five amino acids, but this difference does not prevent the targeting of *yfp* transcripts in RNAi experiments against *gfp*.) In the nerve ring region, where fluorescence intensity was the strongest initially, the signal was reduced by about 40% in $P_{unc\text{-}119}$*sid-1* worms (TU3270); control worms had virtually no reduction in fluorescence intensity (**Fig. 1a**). In the ventral cord and the anterior touch receptor neurons (TRNs), we observed an even greater *sid-1*–dependent reduction in fluorescence intensity (**Fig. 1a**). The posterior TRNs (PLML/R) showed little or no reduction in fluorescence intensity (**Fig. 1a**). We obtained similar results with the TU3311 strain.

Neuronal RNAi in $P_{unc\text{-}119}$*sid-1* worms also produced behavioral defects. To test for specific behavioral effects in a subset

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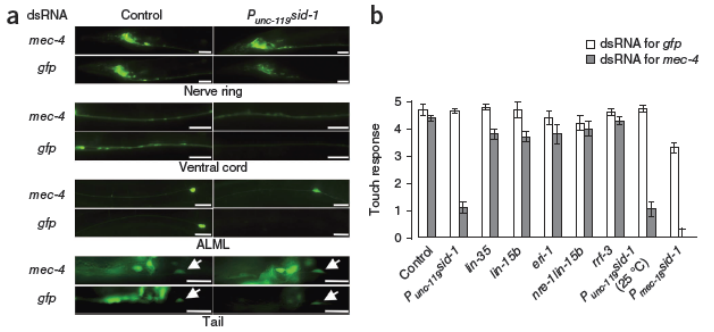

Figure 1 | Expression of *sid-1* in neurons enhances neuronal RNAi. (**a**) Neuronal YFP fluorescence intensity in different areas of $P_{unc-119}$*sid-1*–expressing worms (TU3270) and non-$P_{unc-119}$*sid-1*–expressing worms (TU3310) fed with bacteria producing dsRNA for *gfp*. Both strains contain $P_{unc-119}$*yfp*. Worms were also fed *mec-4* dsRNA, which was not expected to alter fluorescence. Arrows indicate PLML neurons. Scale bars, 25 µm (except images of the ALML neurons, 10 µm). (**b**) Touch-sensitivity (number of responses from five anterior touches) of worms of the indicated genotypes or expressing the indicated transgenes, fed with dsRNA either for *mec-4* or *gfp*. Error bars, s.e.m.; $n = 4$ (each with 30 adult worms), except for the *mec-18* data, $n = 9$ (each experiment with 20 adult worms).

of neurons (the TRNs), we fed worms dsRNA for the TRN channel gene *mec-4* and compared the touch response of $P_{unc-119}$*sid-1* worms to that of wild-type worms and of strains with mutations known to enhance neuronal RNAi (*lin-35*, *lin-15b*, *eri-1*, *rrf-3* and *nre-1 lin-15b*)[11–15]. *mec-4* RNAi produced a marked reduction in the anterior touch response in $P_{unc-119}$*sid-1* worms compared to that in wild-type, *lin-35(n745)*, *lin-15b(n744)*, *eri-1(mg366)*, *nre-1(hd20) lin-15b(hd126)* and *rrf-3(pk1426)* mutants (**Fig. 1b**), although the reduction of response to posterior touch was much smaller (one out of five touches versus three to four out of five touches). We observed the same result in TU3270 and TU3311 worms. As noted above, a similar anterior-posterior difference occurred upon addition of dsRNA for *gfp*. This difference may reflect differential accessibility of dsRNA or differential expression of the wild-type *sid-1* gene (*sid-1(+)*) in the posterior cells, or an intrinsic difference between these two types of neurons.

We observed the same effect of *mec-4* RNAi on the touch response when we grew $P_{unc-119}$*sid-1* worms at 25 °C (**Fig. 1b**). This is a considerable advantage because all other RNAi-hypersensitive strains become either sterile or have severe morphological defects at 25 °C (unpublished observation).

lin-35 and *lin-15b* loss enhances *sid-1* neuronal RNAi

Mutations in *eri-1*, *lin-15b*, *lin-35*, *nre-1* and *rrf-3* improve neuronal RNAi[11–15]. But RNAi in strains with these defects cannot replicate the neuronal phenotype of many genes. For example, the touch insensitivity (Mec) phenotype has been difficult to phenocopy by RNAi; we observed only slight reductions in touch sensitivity with *lin-35(n745)* and *lin-15b(n744)* (**Fig. 1b**; ref. 12). In contrast, YFP fluorescence intensity and touch sensitivity were greatly reduced in strains with $P_{unc-119}$*sid-1* and mutations in *lin-15b* or *lin-35* (**Fig. 2**) upon treatment with dsRNA for *gfp* or *mec-4*, with the greatest reduction in $P_{unc-119}$*sid-1;lin-15b* worms. The Mec phenotype of these strains was so strong that we could see defects in posterior touch; $P_{unc-119}$*sid-1;lin-35* worms responded to three of five posterior touches, and $P_{unc-119}$*sid-1;lin-15b* worms responded only once or twice (data not shown). Mutations in *eri-1*, *nre-1* and *rrf-3* did not improve the response to treatment with dsRNA for *gfp* (data not shown) or *mec-4* (**Fig. 2c**). These results suggest that mutations in *lin-35* and *lin-15b* affect RNAi independently of *sid-1*.

To assess neuronal RNAi further in worms with $P_{unc-119}$*sid-1* alone and in combination with *lin-15b(n744)* and *lin-35(n745)*, we screened 12 *unc* genes that are exclusively expressed in neurons (WormBase) but whose mutant phenotype had not been reproduced by feeding RNAi in wild-type worms or in worms with RNAi-enhanced backgrounds[15–17] and three genes for which RNAi phenotypes had only been reported in *rrf-3* mutants[16] (**Table 1**; we were unable, however, to see RNAi effects for these genes in *rrf-3* worms). RNAi in $P_{unc-119}$*sid-1*, $P_{unc-119}$*sid-1;lin-15b* and $P_{unc-119}$*sid-1;lin-35* worms showed similar phenotypes to the loss-of-function mutant phenotypes for seven of the 15 genes. $P_{unc-119}$*sid-1;lin-l5b* worms were consistently the most sensitive of all strains. Usually the RNAi phenotype was detectable in the adult stage (and sometimes earlier) of worms grown on RNAi bacteria from the time of hatching, although in some cases (**Table 1**) only F1 progeny displayed the phenotype.

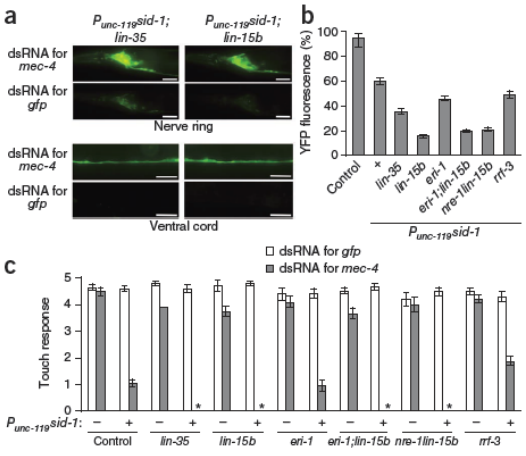

Figure 2 | Mutations in *lin-35* and *lin-15b* enhance RNAi in neurons expressing *sid-1*. (**a**) YFP fluorescence intensity in the nerve ring and ventral cord of worms with the indicated genotypes after feeding with bacteria expressing dsRNA for *gfp* or for *mec-4*. Both strains contain $P_{unc-119}$*yfp*. Scale bars, 25 µm. (**b**) Quantification of YFP fluorescence intensity in the nerve ring of the indicated strains after feeding with bacteria expressing dsRNA for *gfp*, relative to feeding with dsRNA for *mec-4*. Control, strain TU3310, expresses $P_{unc-119}$*yfp* alone. Strains with $P_{unc-119}$*sid-1* were derived from the TU3270 strain and have the *mec-6(+)* transgene. Error bars, s.e.m.; $n = 3$ (each experiment with 30 adult worms). (**c**) Anterior touch response (number of responses from five touches) in worms of the indicated strains fed bacteria expressing dsRNA for *gfp* or *mec-4*. Error bars, s.e.m.; $n = 4$ (each experiment with 30 adult worms). $^*P < 0.05$.

Table 1 | Feeding RNAi for known neuronal genes

Gene	Loss-of-function phenotype[a]	RNAi phenotype[b]						
		$P_{unc-119}$*sid-1*		$P_{unc-119}$*sid-1;lin-35*		$P_{unc-119}$*sid-1;lin-15b*		*lin-15b* or *lin-35* or *nre-1 lin-15b* or *eri-1;lin-15b* or *rrf-3*
unc-13	Lethal	**	++	**	++	**	++	–
unc-14[c]	Severe unc	**	+	**	+	***	+++	–
unc-55[c]	Coiler	**	++	**[d]	++[d]	***	+++	–
unc-58	Mild unc	*	+	*	+	*	+++	–
unc-76	Coiler	*[d]	+[d]	*	+	*	++	–
unc-119	Severe unc; paralyzed	*	++	*	++	*	+++	–
vab-8	Unc	–		*	+	*	+	–
unc-5	Coiler	–		–		–		–
unc-7	Forward kinker	–		–		–		–
unc-10	Coiler	–		–		–		–
unc-17	Lethal	–		–		–		–
unc-24	Kinker	–		–		–		–
unc-30[c]	Shrinker	–		–		–		–
unc-42	Kinker	–		–		–		–
unc-79	Fainter	–		–		–		–

[a]The unc (uncoordinated) phenotype includes a broad category of movement defects, including coiler, kinker, shrinker and paralyzed. Loss of some genes produced lethality. [b]The severity of the RNAi phenotype is described with regard to penetrance (*, a few; **, many; and ***, most) and expressivity (+, weak phenotype compared with the loss-of-function phenotype; ++, intermediate: some worms show a weak phenotype, others have the loss-of-function phenotype; and +++, phenotype is indistinguishable from the loss-of-function phenotype). RNAi for *unc-13* produced slow-growing, slow-moving coilers. –, strains with no RNAi-induced phenotype. [c]Feeding RNAi phenotypes have been reported for these genes when an *rrf-3* mutation is present[11]. [d]The phenotype was seen in the F1 progeny, but not in the worms themselves grown on the indicated bacteria.

As an additional test for the efficiency of neuronal RNAi in $P_{unc-119}$*sid-1* worms, we performed feeding RNAi for 12 *mec* genes needed for touch sensitivity[18]. Feeding RNAi did not reduce touch sensitivity in wild-type worms for any of the genes, and it caused moderate or no reduction in *lin-35* and *lin-15b* worms (**Fig. 3**). In contrast, feeding RNAi resulted in considerable loss of touch sensitivity in $P_{unc-119}$*sid-1* worms for all the genes except *mec-5* (**Fig. 3**), whose expression is needed in muscle cells (B. Coblitz and M.C., unpublished data). The phenotypes were even more severe in $P_{unc-119}$*sid-1;lin-15b* worms (**Fig. 3**). Despite the fact that the transgenic strains contained additional wild-type *mec-6*, feeding with bacteria expressing dsRNA for *mec-6* caused touch insensitivity.

In other work we used DNA microarray analysis of isolated embryonic TRNs to identify 198 genes that are overexpressed in these cells (I.T. and M.C., unpublished data). Using feeding RNAi and $P_{unc-119}$*sid-1;lin-15b* worms, we tested 149 of the 186 genes for which TRN phenotypes were not known and obtained partial touch-insensitive phenotypes for only five of them (*alr-1*, *C03A3.3*, *F46C5.2*, *K11E4.3* and *Y113G7A.15*). We examined worms with mutations in two of these genes. A loss-of-function allele (*oy42*) of the *C. elegans* aristaless gene *alr-1* phenocopied the variable RNAi–induced touch-insensitive phenotype (worms responded to two to seven out of ten touches). A deletion allele of the paraoxonase homolog *K11E4.3* (*ok2266*) produced touch-insensitivity in sensitized backgrounds (Y. Chen and M.C., unpublished data). Given the high efficiency of RNAi in $P_{unc-119}$*sid-1; lin-15b* worms for known *mec* genes, these results indicate that most of the 144 nonresponsive genes are likely not to be essential for touch sensitivity; they are likely to function elsewhere in the TRNs or be redundant.

To test whether we could achieve RNAi by selectively expressing *sid-1* in specific neurons, we expressed *sid-1* under the control of the TRN-specific *mec-18* promoter (P_{mec-18}*sid-1*) from a stable extrachromosomal array in strain TU3312. Worms fed *mec-4* dsRNA were touch-insensitive in the anterior (**Fig. 1b**). Worms fed bacteria making *mec-12* dsRNA were touch-insensitive in the posterior as well: for five anterior touches ($n = 4$ plates of 10 worms), 0.7 ± 0.1 responses and for 10 touches alternatively to the anterior and posterior ($n = 4$ plates of 10 worms), 1.9 ± 0.2 responses. These results indicate that neuronal RNAi can be induced by expressing *sid-1* in specific neurons with an appropriate promoter.

Neuronal expression of *sid-1* decreases nonneuronal RNAi

We noticed a marked reduction of RNAi phenotypes in $P_{unc-119}$*sid-1* worms in response to feeding of dsRNAs expected to act

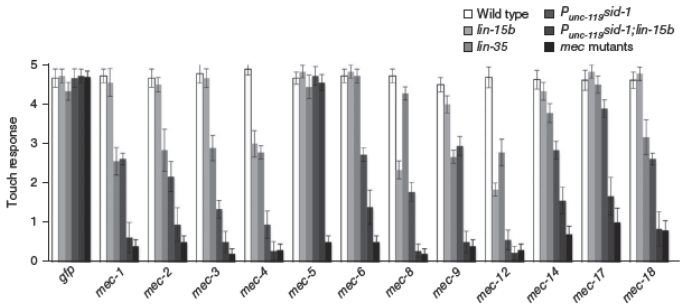

Figure 3 | Enhanced RNAi for genes needed for touch sensitivity. Anterior touch response (out of five touches) in worms of the indicated genotypes fed bacteria expressing dsRNA for known *mec* genes (which give a touch-insensitive phenotype when mutated). *mec* mutants were examined for comparison. Strains with $P_{unc-119}$*sid-1* were derived from TU3270 strain and have the *mec-6(+)* transgene. Error bars, s.e.m.; $n = 9$ (each experiment with 20 adult worms) except for the *mec* mutants, $n = 20$ adult worms.

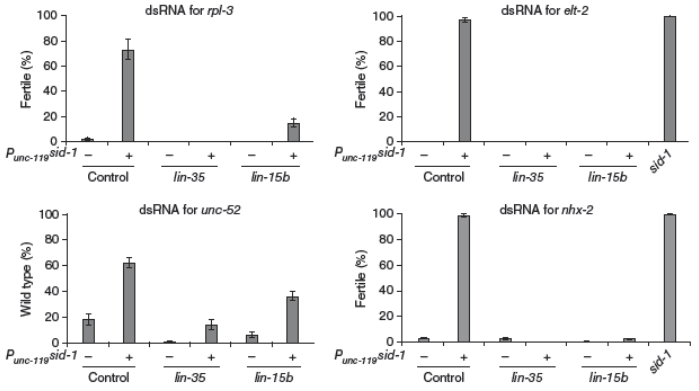

Figure 4 | Expression of *sid-1* in neurons decreases RNAi responses in nonneuronal tissues. The fraction of worms of the indicated genotypes that resisted treatment with dsRNA for various genes (*rpl-3*, *unc-52*, *elt-2* and *nhx-2*) is plotted. For *rpl-3* we counted the number of worms that reached adulthood and became fertile, for *unc-52* we counted the number of paralyzed worms and for *elt-2* and *nhx-2* we counted the number of fertile F1 worms. We used wild type (N2) as controls that do not express $P_{unc-119}$*sid-1*(+) and strain TU3270 as controls that do. *sid-1* mutants were included as negative controls. Error bars, s.e.m.; $n = 9$ (nine plates, 50 worms scored per plate).

in nonneuronal tissues, including *unc-22* (muscle), *rpl-3* (ubiquitous), *unc-52* (hypodermis) and *elt-2* nd *nhx-2* (intestine). In all cases $P_{unc-119}$*sid*-1 worms showed little or no RNAi phenotype in response to RNAi (**Fig. 4**), suggesting a generalized refractory effect to dsRNA in tissues other than neurons. The lesser effect for *unc-52* may reflect SID-1 activity from the *unc-119* promoter, which is expressed in the hypodermis of embryos[19]. All three $P_{unc-119}$*sid-1* strains, TU3270, TU3311 and TU3356, were similarly refractory to nonneuronal RNAi (data not shown). This could be explained by an increased uptake of dsRNA into neurons at the expense of other tissues, causing the dsRNA in those cells to be limiting. The block to nonneuronal RNAi did not occur in the P_{mec-18}*sid-1* strain (data not shown), in which *sid-1* is expressed only in the TRNs, suggesting that this limited expression does not generate a sufficient neuronal reservoir to inhibit the response of nonneuronal cells. The block also did not occur in the $P_{unc-119}$*sid-1* lines with the *lin-35* or *lin-15b* mutations (**Fig. 4**), suggesting that a small amount of dsRNA does enter the nonneuronal cells in $P_{unc-119}$*sid-1* worms but that its activity needs to be amplified by the loss of *lin-15b* or *lin-35* for a phenotype to be seen.

The lack of a response in the intestine, which confirms similar findings[20], is curious because uptake of dsRNA from the intestinal lumen, which requires the *sid-2* gene[20], might be thought sufficient for intestinal RNAi. The requirement for SID-1 in the intestine could indicate that SID-1 is needed either for the initial transport through the intestine or for RNAi in the intestine, perhaps by mediating transport of dsRNA from the pseudocoelom. The finding that RNAi is observed when *sid-1* mutants express *sid-1(+)* in muscle[21] or in the TRNs suggests that SID-1 is not needed for the initial transport of dsRNA from the intestinal lumen and that RNAi in the intestine requires *sid-1*–mediated transport from the pseudocoelom.

Detection of neuronal effects of lethal genes

RNAi experiments for several genes result in a lethal phenotype, making the analysis of specific gene function in neurons difficult or impossible. Because nonneuronal RNAi is blocked in $P_{unc-119}$*sid-1* worms, neuronal phenotypes might be uncovered for lethal genes. We tested this hypothesis by examining six genes (*pat-2* (encoding α-integrin), *pat-3* (encoding β-integrin), *pat-4* (encoding integrin-linked kinase), *pat-6* (encoding actopaxin), *unc-97* (encoding PINCH) and *unc-112* (encoding MIG2)) needed for integrin signaling that encode proteins expressed in both muscle and the TRNs and that we have previously speculated may be involved in touch sensitivity[22]. Mutants with defects in these genes exhibit a severe pat (paralyzed, arrested elongation at the twofold embryo stage) phenotype, making testing of a role in touch-sensitivity difficult.

Wild-type worms fed dsRNA for *pat-2, pat-4, pat-6, unc-97* and *unc-112* became severely paralyzed and sterile, and those fed dsRNA for *pat-3* were arrested as larvae. Those eggs that were fertilized died, confirming the lethal phenotype. However, similarly fed $P_{unc-119}$*sid-1* worms were not paralyzed and did move when prodded with a platinum wire, so their touch sensitivity could be assessed. The $P_{unc-119}$*sid-1* worms were severely touch insensitive (**Fig. 5a**); moreover, they were uncoordinated, suggesting that several types of neurons were affected, and those fed dsRNA for *pat-3* showed a developmental delay. In contrast, feeding RNAi of $P_{unc-119}$*sid-1*

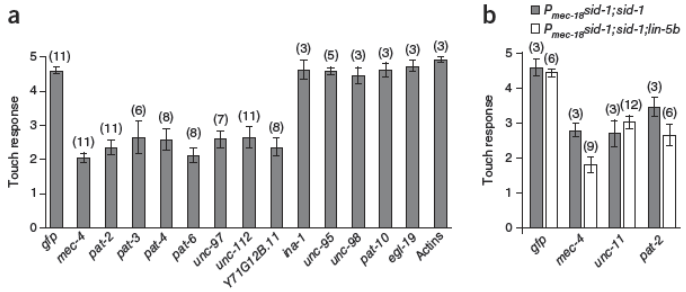

Figure 5 | Eliminating integrin signaling proteins by RNAi in neurons. (**a**,**b**) Touch response (out of five touches) of $P_{unc-119}$*sid-1*–expressing worms (**a**), P_{mec-18}*sid-1; sid-1* worms and P_{mec-18}*sid-1; sid-1; lin-5b* worms (**b**) fed bacteria expressing dsRNA for the indicated genes. In **a**, combined results for three strains (TU3270, TU3311 and TU3401) are shown, because all gave similar results. Error bars, s.e.m.; n = number of plates indicated in the graphs in parentheses (20 adult worms were scored on each plate). The number of plates for each condition is indicated in parentheses.

worms with dsRNA for *unc-95* (encoding paxillin), *ina-1* (encoding α-integrin) and several other genes identified as being needed for muscle dense body function, did not result in touch insensitivity (**Fig. 5a**). These data suggest that integrin signaling is necessary for touch sensitivity.

Because of the pleiotropic phenotype of $P_{unc\text{-}119}$*sid-1* worms fed dsRNA for integrin-signaling genes, we generated a strain in which *sid-1* was only expressed in the TRNs, by expressing $P_{mec\text{-}18}$*sid-1(+)* in *sid-1* mutant worms. When these worms were fed dsRNA for *pat-2* and *unc-112* (**Fig. 5b**), the only phenotype we observed was touch insensitivity, and this was slightly enhanced in worms that also contained a *lin-15b* mutation (**Fig. 5b**). These results demonstrate the possibility of having neuron-specific feeding RNAi. Similar results had been previously obtained in muscle[21]; we extended these observations to show that selective feeding RNAi can be engineered in other cells.

DISCUSSION

Expression of *sid-1* made neurons more susceptible to feeding RNAi, suggesting that the poor response to systemic RNAi in wild-type neurons is due to insufficient SID-1 in most of the nervous system. (As mutations in other genes enhanced feeding RNAi in some neurons, a low level of SID-1 or an equivalent protein is probably present in the wild-type neurons.) Strains expressing SID-1 in neurons could be used to reveal neuronal phenotypes and identify neuronal functions for many genes. An advantage of using the $P_{unc\text{-}119}$*sid-1* strain to reveal neuronal phenotypes by feeding RNAi is that the nervous system and behavior of the worms appear to be similar to those of the wild type, even at 25 °C, whereas other RNAi-enhancing strains would be sterile or dead at the higher temperature (unpublished observations). The poor response to RNAi of neurons in wild-type worms, however, may not be due solely to the lack of SID-1, as we could enhance RNAi through loss of *lin-35* or *lin-15b*. These additive effects indicate separate roles for SID-1 and the loss of LIN-15B and LIN-35. *lin-35* and *lin-15b* mutations may increase the sensitivity of all tissues to dsRNA, whereas SID-1 expression in the nervous system enables the entry of dsRNA to these cells.

We expected that *eri-1* and *rrf-3* mutations would enhance neuronal RNAi in $P_{unc\text{-}119}$*sid-1* worms, but we did not observe this. Moreover, despite reports showing that *eri-1* and *rrf-3* mutants are more sensitive to neuronal RNAi[11,13], we have not observed touch-sensitivity phenotypes upon feeding dsRNA for *mec* genes in these backgrounds (A.C., D.C. and M.C., unpublished data). The lack of neuronal RNAi in *eri-1* and $P_{unc\text{-}119}$*sid-1; eri-1* worms could be due to the restricted nature of *eri-1* expression to the gonad and an unidentified subset of neurons in the head and tail[13], as enhancement would be expected only in the cells expressing *eri-1*. We do not know why loss of *rrf-3* did not give a strong RNAi phenotype in our hands with or without $P_{unc\text{-}119}$*sid-1*, because *rrf-3* is more generally expressed (http://gfpweb.aecom.yu.edu/qgene?0=rrf-3&request=0&request=1&request=2&request=5&request=7&request=8&request=9&9=0&10=0&12=0&13=0&14=0&file=0) and its loss is known to enhance RNAi for some neuronal genes[11]. Perhaps this gene is underexpressed in the TRNs.

The discovery that overexpression of *sid-1* in neurons prevents RNAi in nonneuronal tissues is consistent with the idea that the amount of dsRNA available in the worm for silencing a particular target gene is limited[23]. Moreover, this silencing enabled us to use the $P_{unc\text{-}119}$*sid-1* strain to uncover a requirement for integrin signaling in the TRNs. We obtained similar results in *sid-1* mutants expressing *sid-1(+)* in the TRNs. In general, *sid-1(+)* transgenes expressed from panneuronal or neuron-specific promoters, especially in backgrounds such as *sid-1;lin-15b*, which would enhance RNAi only in cells expressing the transgene, should be useful in uncovering neuronal phenotypes for other genes that are widely expressed or exhibit considerable pleiotropy or lethality. Other tissue-specific promoters could also be used for *sid-1(+)* expression, to allow for selective feeding RNAi in other cells.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

All authors designed experiments; D.C. generated the initial constructs and constructed the multiply mutant lines; X.C. generated the cell-specific constructs and constructed strains with the *sid-1* mutation; A.C., D.C., I.T. and X.C. conducted the RNAi tests; and A.C., I.T. and M.C. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

***C. elegans* growth.** Wild-type *C. elegans* (N2) and strains with mutations affecting RNAi (*lin-35(n745)I*[12], *rrf-3(pk1426)II*[16], *eri-1(mg366)IV*[13], *sid-1(qt2)V*[5], *sid-1(pk3321)V*[24], *lin-15b(n744)X*[25], *eri-1(mg366)IV;lin-15b(n744)X*[17] and *nre-1(hd20) lin-15b(hd126)X*[14]), mutations causing touch insensitivity (refs. 18,26 except were noted; *mec-1(e1496)V, mec-2(u37)X, mec-3(e1338)IV, mec-4(u253)X, mec-5(u444)X, mec-6(u450)I, mec-8(e398)I, mec-9(u437)V, mec-10(ok1104)X* (*C. elegans* Gene Knockout Consortium), *mec-12(e1605)III, mec-14(u55)III, mec-17(tm2109)IV* (National Bioresource Project, Japan), *mec-18(u69)X)* or mutations causing uncoordination (*unc-5(e53)IV, unc-7(e5)X, unc-10(e102)X, unc-24(e138)IV, unc-30(e191)IV, unc-42(e270)V, unc-55(e1170)I, unc-79(e1068)III, unc-14(e57)I, unc-76(e911)V)*[27], *vab-8(e1017)V*[28] and *unc-119(e2498)III*[29]) were grown at 20 °C as previously described[27].

Expression constructs and transformation. A 2.2-kb fragment 5′ from the start of translation of the *unc-119* gene was amplified from genomic DNA introducing 5′ HindIII and 3′ BamHI sites, and cloned into TU739 (ref. 30) to create TU865 ($P_{unc\text{-}119}yfp$). A 7.8-kb fragment 5′ from the start of translation of the *sid-1* gene was similarly amplified from genomic DNA introducing 5′ BamHI and 3′ NotI sites and cloned into BamHI-EagI sites of TU864 to create TU866 ($P_{mec\text{-}18}sid\text{-}1$). A BamHI and PvuI digested fragment of TU866 containing *sid-1* was cloned into TU865 to create TU867 ($P_{unc\text{-}119}sid\text{-}1$). Primers used are listed in **Supplementary Table 1**.

We generated transgenic worms by microinjection with one or more of the above plasmids[31], isolating worms with extrachromosomal arrays and integrating the subsequent extrachromosomal arrays with gamma rays (4,800 rad)[30]. All plasmids, including markers, were injected at a concentration of 25–30 ng μl^{-1} except for TU866, which was injected at 5 ng μl^{-1}, $P_{mec\text{-}6}mec\text{-}6$, which was injected at 2 ng μl^{-1}, and pBSK (Stratagene) added to reach a concentration of 100 ng μl^{-1}.

Enhanced RNAi strains. *uIs57* contains $P_{unc\text{-}119}sid\text{-}1$, $P_{unc\text{-}119}yfp$ *and* $P_{mec\text{-}6}mec\text{-}6$ integrated into wild-type worms (TU3270). *uIs57* was crossed into several mutations to create strains TU3272 (*lin-35 (u745)*), TU3335 (*lin-15b(u744)*), TU3337 (*eri-1(mg366)*), TU3339 (*rrf-3(pk1426)*), TU3341 (*eri-1(mg366); lin-15b (n744)*) and TU3344 (*nre-1(hd20) lin-15(hd126)*).

TU3310 contains *uIs59*, an integrated array of $P_{unc\text{-}119}yfp$, which expresses YFP in all neurons, and TU3311 contains *uIs60*, which is an integrated array of $P_{unc\text{-}119}sid\text{-}1$ and $P_{unc\text{-}119}yfp$, which expresses YFP and SID-1 in all neurons.

uEx762 is an extrachromosomal array containing $P_{mec\text{-}18}sid\text{-}1$ and $P_{sng\text{-}1}yfp$, which expresses YFP in all neurons and SID-1 in the TRNs, transformed into N2 worms to create TU3312.

TU3356 contains *uEx766*, an extrachromosomal array containing $P_{unc\text{-}119}sid\text{-}1$ and $P_{unc\text{-}4}mdm2::gfp$ (plasmid TU703 (ref. 32)); this DNA encodes a fusion of GFP and the RING domain of mammalian Mdm2 E3 ubiquitin ligase, which expresses YFP in a subset of motor neurons and SID-1 in all neurons.

uIs69 contains pCFJ90 ($P_{myo\text{-}2}mCherry$) and TU867 ($P_{unc\text{-}119}sid\text{-}1$) integrated into *sid-1(pk3321)* worms (TU3401).

TU3403 is a strain containing *uIs71*, an integrated array of pCFJ90 ($P_{myo\text{-}2}mCherry$)[33] and TU866 ($P_{mec\text{-}18}sid\text{-}1$), and *ccIs4251* (*myo-3::Ngfp-lacZ, myo-3::Mtgfp*); *sid-1(qt2)*). The ccIs4251; *sid-1(qt2)* comes from HC75 (ref. 5). TU3568 is *uIs71; sid-1 (pk3321) him-5(e1490); lin-15b(n744)*.

RNAi by feeding. Bacteria expressing dsRNA, taken from the Ahringer library[4,5] were grown on LB plates supplemented with ampicillin at 37 °C overnight. Next morning a large amount of bacterial lawn was inoculated in LB liquid supplemented with ampicillin and grown for 6–8 h. The resulting culture was seeded onto 1-day-old NGM-IPTG-carbenicillin plates[4] and allowed to dry for 24 or 48 h. We added 20–40 embryos, obtained by bleaching of gravid hermaphrodites, to each plate after the plates dried out and grew them at 15 °C. Worms treated with all dsRNA used in this work, were examined as adults 5 d after the embryos were added to the plates, at 15 °C. Worms fed with dsRNA for *mec-4, gfp, elt-2, nhx-2* and the 15 neuronal genes, were also scored as adults in the next generation (F1). For the quantification of RNAi mediated by dsRNAs for *mec-4* and *gfp*, F1 worms were transferred as L1 larvae and then again as L4 larvae to fresh RNAi plates and then scored 36 h later.

Most of the fluorescent markers contained the coding region for *yfp*, which differs from *gfp* in eight nucleotides: 193 (thymine in *gfp*, cytosine in *yfp*), 202 (guanine, cytosine), 214 (thymine, guanine), 216 (guanine, cytosine), 239 (guanine, adenine), 607 (adenine, thymine), 608 (cystosine, adenine) and 609 (adenine, cytosine). These changes result in five amino acid changes in from GFP to YFP (C65G, V68L, S72A, R80Q and T203Y). These differences do not prevent the targeting of *yfp* transcripts in RNAi experiments by feeding worms with dsRNA for *gfp* (pPD128.110 from the Fire vector collection).

Microscopy. YFP fluorescence and differential interference contrast were observed using a Zeiss Axiophot II. All photographs of worms subjected to RNAi were taken with a Diagnostic Instruments Spot 2 camera using a Plan NEOFUAR 25× objective, for 300 ms at a gain of 1. YFP intensity was quantified using ImageJ (http://rsb.info.nih.gov/ij/). A fixed area of 200 (width) × 500 (length) pixels was measured from the tip of the nose, which covered the entire nerve ring.

Touch sensitivity. Twenty to thirty adult worms (36 h after L4 stage at 15 °C) were touched gently with an eyebrow hair[26] ten times with alternative anterior and posterior touches (five each) to determine an average response. These experiments were repeated several times to obtain a mean and s.e.m. Because the effects on anterior touch were stronger in these experiments, we usually reported only those responses. Only worms that moved when prodded with a platinum wire and that looked normal were assayed.

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Appendix II. List of *C. elegans* strains and PCR primers

***C. elegans* strains**

strain name	*genotype*
TU3595	*sid-1(pk3321) him-5(e1490) V; lin-15B(n744) X; uIs72*
TU3568	*sid-1(pk3321) him-5(e1490) V; lin-15B(n744) X; uIs71*
RB1887	*tom-1(ok2437) I*
VC2446	*+/mT1 II; cdk-1(ok1881)/mT1pdpy-10(e128)] III*
VC3455	*kin-18(ok395) III/hT2[bli-4(e937) let-?(q782) qIs48](I;III)*
VC556	*tag-170&vps-16(ok776) III/hT2[bli-4(e937) let-?(q782) qIs48] (I;III).*
VC703	*ani-2(ok1147)/mIn1[mIs14 dpy-10(e128)] II.*
WM74	*wrm-1(ne1982) III*
CZ4380	*ifb-1(ju71) II*
DG1856	*goa-1(sa734) I*
EU1135	*tba-1(or346) I*
GS2526	*arIs37 I; mca-3(ar492)dpy-20(e1282) IV*
MJ70	*emb-9(hc70) III*
FX3828	*F59E12.11(tm3828) II*
TU4272	*uIs109 III; mca-3(ar492)dpy-20(e1282) IV*
TU4273	*mca-3(ar492)dpy-20(e1282) IV; uIs113*
TU3842	*uIs109 III; him-5(e1490) V*
TU3836	*pat-2(ok2148); uEx829*
TU3689	*pat-3(st564; uEx826(Ppat-3::pat-3, pCFJ90, Pmec-3::RFP)*
TU3834	*pat-6(st561); uEx827(Ppat-6::pat-6, pCFJ90, Pmec-3::RFP)*
TU3687	*unc-112(gk1); uEx824(Pu112::u112, pCFJ90, Pmec-3RFP)*
TU3839	*unc-97(ra115)dpy-8(e130); uEx832*
TU253	*mec-4(u253)*
TU3851	*uIs91 III; sid-1(pk3321) him-5(e1490) V*
TU3850	*pat-2(ok2148); uIs94; uEx830*
TU3859	*pat-3(st564) III; uIs94; uEx826*
TU3853	*uIs91 III; pat-6(st561) IV; uEx827*
TU3854	*uIs91 III; unc-97(ra115)dpy-8(e130) X; uEx832*
TU3852	*uIs91 III; unc-112(gk1) V; uEx824*
TU3855	*uIs91 III; egl-19(ad1013) IV; sid-1(pk3321) him-5(e1490) V*
TU4274	*uIs156*
NG144	*ina-1(mg144) III*
NG39	*ina-1(mg39) III*
TU4275	*uIS109 III; egl-19(ad1006) IV*
TU3844	*pat-2(ok2148) uIs109 III; uEx829*
TU4277	*uIs109 III; unc-112(gk1); UEx862*
JK1438	*daf-2(m65)/qC1 dpy-19(e1259) glp-1(q339) III*
CY401	*sqt-1(sc13) age-1(mg109)/mnC1 dpy-10(e128) unc-52(e444) II*

JT9609	*pdk-1(sa680) X*
RB759	*akt-1(ok525) V*
VC204	*akt-2(ok393) X*
GR1307	*daf-16(mgDf50) I*
RB712	*daf-18(ok480) IV*
GR1318	*pdk-1(mg142) X*
GR1310	*akt-1(mg144) V*
CB1370	*daf-2(e1370)III*
HT1890	*daf-16(mgDf50) I; daf-2(e1370) III*
DR1309	*daf-16(m26) I; daf-2(e1370) II*
DR1564	*daf-2(m41) III*
PJ1146	*daf-2(m41) III; ccIs55 V; pdk-1(mg142) X*
DR1942	*daf-2(e979) III*
TU4276	*akt-1(mg144) unc-112(gk1) V; uEx862*
TU4278	*unc-112(gk1) V; pdk-1(mg142) X; uEx862*
TU4279	*daf-16(mgDf50) I; unc-112(gk1) V; uEx862*
TU4280	*pat-2(ok2148) uIs109 III; akt-1(mg144)*
TU4281	*pat-2(ok2148) uIs109 III; pdk-1(mg142)*
TU45	*mec-4(u45) X*
TU4282	*akt-1(mg144) V; mec-4(u45) X*
TU4283	*daf-16(mgDf50) I; mec-4(u45)X*
VC708	*mfb-1(gk311) I*
BC14180	*dpy-5(e907) I; sIs13037*
TU4284	*mfb-1(gk311) I; akt-1(ok525) V*
TU4285	*akt-1(ok525) V; sIs13037*
RV110	*uba-1(it129) IV*
TU4286	*uba-1(it129) IV; akt-1(ok525) V*
RB1679	*cav-1(ok2089) IV*
TU4287	*cav-1(ok2089) IV; akt-1(ok525) V*
CB1267	*him-4(e1267)X*
TU4289	*him-4(e1267) uIs157 X*
FX2988	*ins-33(tm2988) I*
FX3498	*ins-10(tm3498) V*
FX4990	*ins-22(tm4990) III*
TU3927	*uIs126*
TU3929	*uIs128*
TU4290	*uIs109 III; uIs126*
TU4291	*uIs109 III; uIs128*
TU4292	*akt-1(mg144) V; uIs128*
TU4293	*pdk-1(mg142) X; uIs128*
TU4294	*uIs157 X; uIs 128*

TU4295	*uIs155*
TU4296	*uIs157*
OH3679	*che-1(ot151) otIs114 I*
TU4297	*che-1(ot151) otIs114 I; uIs157 X*

Integrated and extra-chromosomal arrays

uIs71	*[pCFJ90(myo-2p::mCherry), mec-18p::sid-1]*
uIs72	*[pCFJ90(myo-2p::mCherry), unc-119p::sid-1, mec-18p::mec-18::GFP]*
uIs109	*[mec-17p::gcamp3, ceh-22p::gfp] III*
uIs113	*[mec-3p::gcamp3, mec-3p::rfp]*
uIs91	*[mec-4p::ChR2::yfp, mec-17p::gfp, myo-3p::mcherry] III*
uIs04	*[mec-4p::ChR2::yfp, mec-17p::gfp, myo-3p::mcherry]*
uIs157	*[mec-3p::pdk-1(mg142), myo-2p::mcherry] X*
uIs152	*[mec-3p::rfp]*
uIs128	*[ins-10p::ins-10(i), ins-10p::fp]*
uIs126	*[ins-10p::ins-10(i), ins-10p::fp]*
uIs156	*[unc-112p::unc-112::gfp, myo-2p::mCherry]*
uEx824	*unc-112p::unc-112, pCFJ90, mec-3p::rfp*
uEx825	*unc-112p::unc-112, pCFJ90, mec-3p::rfp*
uEx826	*pat-3p::pat-3, pCFJ90, mec-3p::rfp*
uEx827	*pat-6p::pat-6, pCFJ90, mec-3p::rfp*
uEx828	*pat-6p::pat-6, pCFJ90, mec-3p::rfp*
uEx829	*pat-2p::pat-2, pCFJ90, mec-3p::rfp*
uEx830	*pat-2p::pat-2, pCFJ90, mec-3p::rfp*
uEx831	*unc-97p::unc-97, pCFJ90, mec-3p::rfp*
uEx832	*unc-97p::unc-97, pCFJ90, mec-3p::rfp*
uEx833	*unc-97p::unc-97, pCFJ90, mec-3p::rfp*
uEx862	*unc-112p::unc-112, pCFJ90, mec-3p::rfp*

Primers for cloning and genotyping

name	sequence	note
tag170res_f	ccacacagaggtcttcggcatc	*tag-170* rescue
tag170res1_r	ggagcatgagactcccccatttc	*tag-170* rescue
ATGGCaMP3_B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAATGCGGGGTTCTCATCATCATCA	GCaMP3
ATGGCaMP3_B2_ rev1	GGGGACCACTTTGTACAAGAAAGCTGGGT GTCACTTCGCTGTCATCATTTGTACAAACT	GCaMP3
Pm18ATG-B4	GGGGACAACTTTGTATAGAAAAGTTGAAT TAATTCGTCTACTATCCACGTGTCGAT	*mec-18* promoter
Pm18ATG-B1r_rev	GGGGACTGCTTTTTTGTACAAACTTGCCAT	*mec-18* promoter

	GCTCACAACCTTCTTGGAAGGCG	
5UTR-B3_rev	GGGGACAACTTTGTATAATAAAGTTGGGA AACAGTTATGTTTGGTATATTGGGAATG	*unc-54* 3'UTR into gateway vectors
STOP-5UTR-B2r	GGGGACAGCTTTCTTGTACAAAGTGGTTT AGCATTCGTAGAATTCCAACTGAGCG	*unc-54* 3'UTR into gateway vectors
IVS-GYCFP-5UTR-B2r	GGGGACAGCTTTCTTGTACAAAGTGGTTT GGCCAAAGGACCCAAAGGTATG	gfp + *unc-54* 3'UTR into gateway vectors
IVS-RFP-5UTR-B2r	GGGGACAGCTTTCTTGTACAAAGTGGTTT TGGCCAAAGGACCCAAAGGTATG	mTagfrp + *unc-54* 3'UTR into gateway vectors
IVS-GYCFP-B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTATGGCCAAAGGACCCAAAGGTATG	gfp into gateway vectors
IVS-GYCFP-B2_rev	GGGGACCACTTTGTACAAGAAAGCTGGGT GTTTGTATAGTTCATCCATGCCATGTGTAA TC	gfp into gateway vectors
IVS-RFP-B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTATTGGCCAAAGGACCCAAAGGTATG	rfp into gateway vectors
IVS-RFP-B2_rev	GGGGACCACTTTGTACAAGAAAGCTGGGT GATTAAGTTTGTGCCCCAGTTTGCTAGG	rfp into gateway vectors
unc-112_B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAGCACATCTTGTTGAAGGAACCTCGA	*unc-112* coding region
unc-112_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGT GAGCCCATCCTCCTGTAAGTTTGTGG	*unc-112* coding region
pat-2_B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTACGAGAGGGTAGTTTTCCGCGAAG	*pat-2* coding region
pat-2_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGT GTAGCATTTGTCCGTGACGTCCCT	*pat-2* coding region
pat-3_B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTACCACCTTCAACATCATTGCTGCTC	*pat-3* coding region
pat-3_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGT GGTTGGCTTTTCCAGCGTATACTGGA	*pat-3* coding region
unc97-B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAGATTCCGACCACAACCATATCAACG	*unc-97* coding region
unc97-B2-r	GGGGACCACTTTGTACAAGAAAGCTGGGT GTTTTGGTCCAGGACTCATCGATCTTC	*unc-97* doing region
Pu112_B4	GGGGACAACTTTGTATAGAAAAGTTGAGA GGCACTGAAGAAAATGCGAGG	*unc-112* promoter
Pu112_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCcatG TTTGAAGTGATTTAGGTAGAACCACTGA	*unc-112* promoter
Pp2_B4	GGGGACAACTTTGTATAGAAAAGTTGTTT GAATTCTACCATTTCGGTAACTGATAGG	*pat-2* promoter
Pp2_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCcatC TACTGGAAATTTGGAATTCGGTTTTTTG	*pat-2* promoter
Pu97_B4	GGGGACAACTTTGTATAGAAAAGTTGAAG CTAGTTTTCACTAACAGGAAGATGCTATA AAA	*unc-97* promoter

Pu97_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCcatTCCGCCACACACCATCTATTATTGAA	*unc-97* promoter
Pp3_B4	GGGGACAACTTTGTATAGAAAAGTTGGGTGAACGACCCGAAATTGAGTGA	*pat-3* promoter
Pp3_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCcatTTGATGCCGGGTAGGTTCAACTG	*pat-3* promoter
Ppat-6_B4	GGGGACAACTTTGTATAGAAAAGTTGTTACCCGTTAATGATCAATGAGAGTGG	*pat-6* promoter
Ppat-6_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCcatGGTTATTGCTGAAAAGTTTAAAATTATTAAGGG	*pat-6* promoter
pat-6_B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTATCAACACTTGGTCGTAGTAAGACCCCA	*pat-6* coding region
pat-6_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGGATATGTTTGTACTTAGTGAAAAGCAAATGGAGA	*pat-6* coding region
mg142_seq_forw1	TCGGATGAGGAGGGTAAGGTTTTC	*pdk-1(mg142)* genotyping primers
mg142_seq_rev1	CTCCGGCTCGCCAAATGTG	*pdk-1(mg142)* genotyping primers
mgDf50_seq_forw	CAATGAGCAATGTGGACAGC	*daf-16(mgDf50)* genotyping primers
mgDf50_seq_rev	CCGTCTGGTCGTTGTCTTTT	*daf-16(mgDf50)* genotyping primers
mg144_seqf	GTCGGCAGAAGTTCGTCAGCGT	*akt-1(mg144)* genotyping primers
mg144_seqr	GTGAGCAACTTCTTCGCGAGCAA	*akt-1(mg144)* genotyping primers
Pins22_B4	GGGGACAACTTTGTATAGAAAAGTTGgcgaagtccaataagtttacggtctactg	*ins-22* promoter
Pins22_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCcatttttgttggttagttgtactaggatatcaaagac	*ins-22* promoter
ins22res_B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAgcgaagtccaataagtttacggtctactg	*ins-22* genomic DNA rescue
ins22res_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGtatcgaaattccagaaatccggatgttcg	*ins-22* genomic DNA rescue
Pins10_B1r_r	GGGGACTGCTTTTTTGTACAAACTTGCCATtgttagaagtgctggaaattgtgataaagtc	*ins-10* promoter
Pins10_B4	GGGGACAACTTTGTATAGAAAAGTTGgacggtgggtggagagagtg	*ins-10* promoter
ins10res_f	gacggtgggtggagagagtg	*ins-10* genomic DNA rescue
ins10res_Rev	gaaaggaagtacggtacctagtacctg	*ins-10* genomic DNA rescue
ins10i1_B1_r	GGGGACAAGTTTGTACAAAAAAGCAGGC	*ins-10* hairpin RNAi

	TTAttataaaacggagcagcaggagatcc	construct
ins10i1_B2	GGGGACCACTTTGTACAAGAAAGCTGGGT Gtcactgcatttctccactattcaaaaaaca	*ins-10* hairpin RNAi construct
ins10i1_B2r	GGGGACAGCTTTCTTGTACAAAGTGGTTtc actgcatttctccactattcaaaaaaca	*ins-10* hairpin RNAi construct
ins10i1_B3_r	GGGGACAACTTTGTATAATAAAGTTGtgaaa ggaagtacggtacctagtacctg	*ins-10* hairpin RNAi construct
ok776seq_forw	ttccacacagaggtcttcggc	*tag-170(ok776)* genotyping primers
ok776seq_rev	accgattggacctgagaattggc	*tag-170(ok776)* genotyping primers
ar492seq_forw	ctgtccccaaagaagtgttccaac	*mca-3(ar492)* genotyping primers
ar492seq_rev	gccgtcgtagttgcgttcg	*mca-3(ar492)* genotyping primers
tag170res_f	ccacacagaggtcttcggcatc	*tag-170* genomic DNA rescue
tag170res1_r	ggagcatgagactcccccatttc	*tag-170* genomic DNA rescue
pdk1_B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAatggaggatctcacaccaactaacac	*pdk-1(mg142)* cDNA
pdk1_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGT Gtcaaggcgacttcttgtccatttg	*pdk-1(mg142)* cDNA
ok393_seqr	cgaaatgtgctctacgcaaa	*akt-2(ok393)* genotyping primers
ok393_seqf	aaccgcaactaccaaaatgc	*akt-2(ok393)* genotyping primers
ok525_seqf	ttgagcgaacattctatgcg	*akt-1(ok525)* genotyping primers
ok525_seqr	gtcgtggtgacaagggaagt	*akt-1(ok525)* genotyping primers
tm4639_seq1	CTCCCCAGATTAGAATTGCT	*ins-22(tm4639)* and *ins-22(tm4990)* genotyping primers
tm4639_seq2	AGGGGCTTCTTTGACACGTA	*ins-22(tm4639)* and *ins-22(tm4990)* genotyping primers
ins22res2_B1	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAcacaagattaactggtttggtatcacgag	*ins-22* genomic DNA rescue
ins22res2_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGT Gtcacagcgactgcacttatcaac	*ins-22* genomic DNA rescue
it129_seq1	agagtagcgaatgtatggaacttcgac	*uba-1(it129)* genotyping primers
it129_seqr	agccaagttctggtcaggagc	*uba-1(it129)* genotyping primers

ok2089_seq	ccatttcccatctgttaccg	*cav-1(ok2089)* genotyping primers
ok2089_seqr	tggatgaaagagcacacagc	*cav-1(ok2089)* genotyping primers
gk311_Seq	cctgtatgccgactccttgt	*mfb-1(gk311)* genotyping primers
gk311_Seqr	tgcggtgtaatatgagccaa	*mfb-1(gk311)* genotyping primers
mfb1c_B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGCCATTCATTGGACGTGATTGG	*mfb-1* cDNA
mfb1c_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTACAAATAAATCAACATATCGACAAATTGTCTGGG	*mfb-1* cDNA
cav1_B1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAatgagattgtgcaacgtgtggaatg	*cav-1* genomic coding region
cav1_B2_r	GGGGACCACTTTGTACAAGAAAGCTGGGTGgacgcatggagcagtagtttcttg	*cav-1* genomic coding region

Appendix III. List of important *C. elegans* genes involved and their functions

Gene name	Function
pat-2	α-integrin
pat-3	β-integrin
pat-4	integrin-linked kinase (ILK)
pat-6	actopaxin/parvin
unc-97	PINCH
unc-112	Mig-2/Kindlin
daf-2	insulin receptor
daf-18	PTEN
daf-16	Foxo transcription factor
age-1	phosphatidylinositide 3-kinase (PI3K)
pdk-1	3-phosphoinositide dependent kinase (PDK)
akt-1	protein kinase B (PKB/AKT)
akt-2	protein kinase B (PKB/AKT)
ins-10	insulin-like peptide
ins-22	insulin-like peptide
mfb-1	E3 ubiquitin ligase
uba-1	E1 ubiquitin activating enzyme
cav-1	caveolin
mec-4	DEG/ENaC mechanotransduction channel