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Hierarchy of Habituation Induced by Mechanical Stimuli in *Caenorhabditis elegans*

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ABSTRACT—*C. elegans* becomes habituated to repetitive mechanical stimuli. We compared the habituated states induced by three types of mechanical stimuli: touch on the head (head-touch), touch on the anterior body (body-touch), and mechanical tapping of the Petri dish, all of which evoke backward movement. The habituation patterns were similar, but differed in retention period and/or the rate of recovery. We found a hierarchy between the habituated states induced by the three types of mechanical stimuli in the decreasing order of head-touch, body-touch, and tap stimulus. Evidence is presented that the hierarchy is brought out by the magnitude of stimuli rather than by independent neural pathways.

INTRODUCTION

Learning is a major vehicle for behavioral adaptation in animals (Carew and Sahley, 1986). Experimentally, learning is divided into two classes, associative and nonassociative learning. For associative learning, animals are exposed to different stimuli to learn the relationship of one stimulus to another or the relationship of a stimulus to behavior. For nonassociative learning, animals are exposed once or repeatedly to a single type of stimulus and learn about the properties of the stimulus. Habituation and sensitization are examples of nonassociative learning.

The nematode *C. elegans* has been used for studies of the biological basis of learning and memory and is known to be able to learn both associatively and nonassociatvely (Rankin and Broster, 1992; Jorgensen and Rankin, 1997). To understand the mechanisms of learning and memory, *C. elegans* is a promising organism because its nervous system is extremely simple (Sulston *et al.*, 1983; White *et al.*, 1986; Rankin *et al.*, 1990). *C. elegans* moves backward when subjected to a vibratory stimulus applied through the medium. This response, termed the tap withdrawal reflex, shows habituation because repeated plate tapping stimuli cause a decrease in the magnitude of response. The neural circuitry mediating the tap withdrawal reflex was identified by ablating neurons and by noting the effects of the ablation on the worm's withdrawal reflex (Wicks and Rankin, 1995; Wicks *et al.*, 1996).

Detailed analyses of habituation behavior are expected to reveal how and where memory is retained in the nervous system. One approach is to compare habituations to different stimuli. Habituations evoked by mechanical stimuli have been studied in *C. elegans* (Jorgensen and Rankin, 1997). However, it is not clear how habituated states change with type and strength of stimuli. We adopted and compared three types of mechanical stimuli, all of which evoke withdrawal response. We found the habituation evoked by the different stimuli were not identical but hierarchical. We will discuss that the hierarchy is based on the difference of the magnitude of stimuli.

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MATERIALS AND METHODS

Maintenance of *C. elegans*

Wild-type *C. elegans* were grown and maintained as described by Brenner (1974). Synchronously staged adult hermaphrodites were obtained by growth for 70~80 hr after hatching at 20°C.

Mechanical stimuli

For testing responses to mechanical stimuli, worms were transferred from a culture plate to 3.5 cm Petri plates filled with NGM agar and streaked with *E. coli* OP50. To become familiarized with their new surroundings, worms were allowed to adjust to their surroundings for at least 10 min prior to testing. All tests were performed at 20°C.

The response of *C. elegans* to touch stimuli was tested by lightly stroking across the body with the fine tip of an eyelash. Fig. 1 shows two positions at which touch stimuli were given. A touch stimulus was given as the worm moved slowly forward, and then again when the worm began forward movement. Consequently intervals of touch stimuli were 5~15 sec. To assess the magnitude of responses, the distance covered by the backward movement was measured under a stereomicroscope with an eyepiece graticule. The distance was expressed in body lengths of the worms.

Tap stimuli were given to worms with the modified apparatus and method of Gannon and Rankin (1995). A mechanical tapper

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stroked the side of a Petri dish and the vibrations were transmitted through the plate and agar. The intensity of the tap was controlled by the stimulator; usually the stimulator was set at a 70 μsec pulse. In the cases indicated, 50 or 90 μsec pulses were used. The response magnitude was measured as the distance of backward movement. If not otherwise mentioned, tap stimuli were given to worms at 10-sec interstimulus intervals (10-s ISI).

Cell kills

AVA interneurons were killed with a laser microbeam (Sulston and White, 1980; Bargmann and Avery, 1995). Control worms were treated identically to operated animals, but no cells were killed (mock kills).

Statistical analysis

The data of response magnitude were compared across the groups using ANOVA with Fisher's PLSD post hoc tests (STATVIEW, Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Magnitude of response evoked by mechanical stimuli

C. elegans were given three types of mechanical stimuli: head-touch, body-touch (Fig. 1), and tapping. All stimuli evoked backward movement of worms. The distance of the backward movement differed depending on the type of stimuli (Fig. 2). Worms stimulated by taps moved backward about one-half the distance of touched worms, and these differences were highly significant ($p<0.0001$ in both cases). However, the response magnitude was not significantly different between the two touch stimuli ($p=0.3191$).

Habituation induced by repeated stimuli

Fig. 3 shows habituation patterns of reversal response evoked by 50 serial rounds of mechanical stimuli. With all types of stimuli, the population fell into a habituated state in the early stages of training and attained a steady state. However, the extent of habituation was not identical among the three types of stimuli. That is, the reversal magnitude was within 0.1 in the tap stimulus, though the magnitude was above 0.2 in the head-touch stimulus.

Recovery from habituation

To test the retention of the habituated state, worms were habituated by 50 serial taps of 10-s ISI and kept unstimulated for 5 min. Then the trained worms were tested by 30 serial taps of 10-s ISI. To the first tap of the test stimuli, they responded strongly but fell into the habituated state much more rapidly than the non-trained worms (Fig. 4). We then compared recovery from habituation induced by the three types of mechanical stimuli (Fig. 5). Worms habituated by any mechanical stimulus recovered gradually. Worms trained by touch and 10-s ISI tap stimulations partially recovered from habituation within 10 min with high significancies ($p<0.0001$). Whereas worms trained by 2-s ISI completely recovered from the habituation within 10 min (Fig. 5). The recovery from the habituation under various stimuli is already reported (Broster and Rankin, 1994; Wicks and Rankin, 1996a). Contrary to the result of 2-s and 10-s ISI after the 10 min-retention, significant difference was not observed in the extent recovery between 10-s and 60-s ISI (Broster and Rankin, 1994).

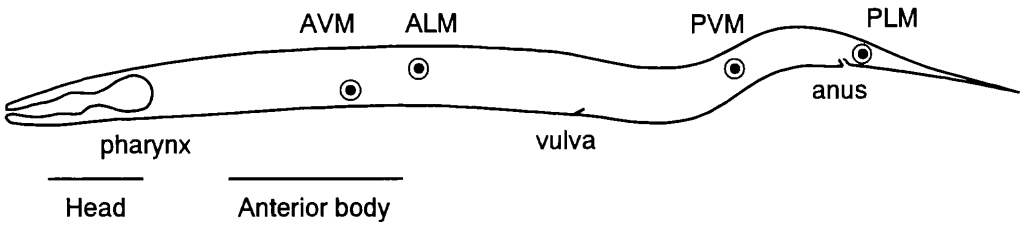

Fig. 1. Schematic drawing of the positions of mechanosensory neurons of the *C. elegans* hermaphrodite. At the indicated head and anterior body regions, touch stimuli were given.

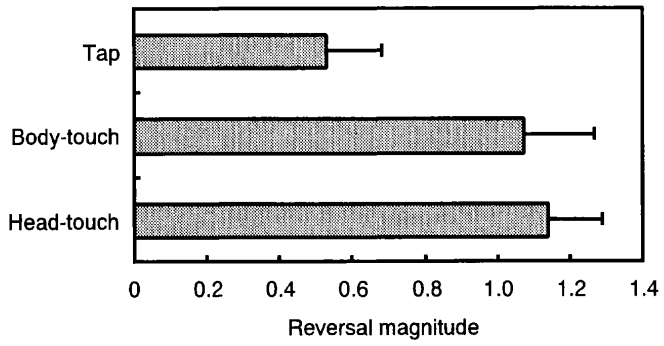

Fig. 2. Response magnitude evoked by the three types of mechanical stimuli. Reversal magnitude was represented as the relative values of the distance of backward movement per body length of worms. Mean values (±SD) of 100 trials using 20 worms are presented.

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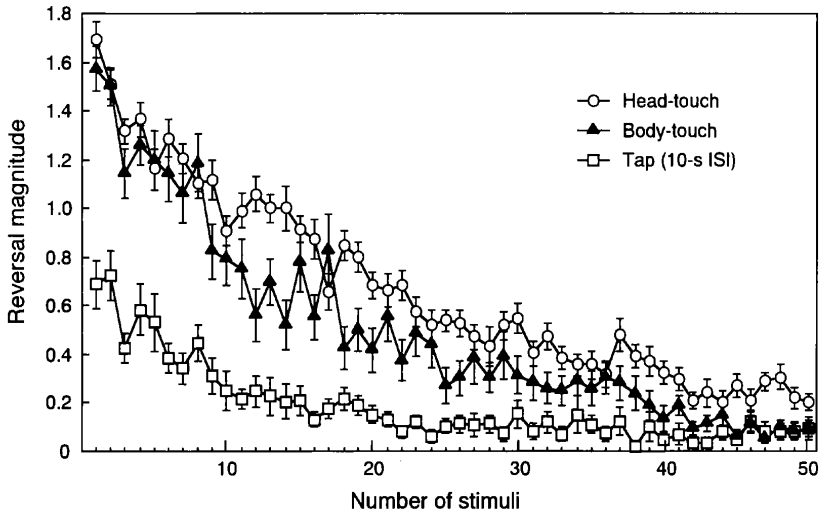

Fig. 3. Habituation curves produced by trains of head-touch (○), body-touch (▲), and tap at 10-s ISI (□). Mean response values (±SE) of stimuli of 50 worms are presented.

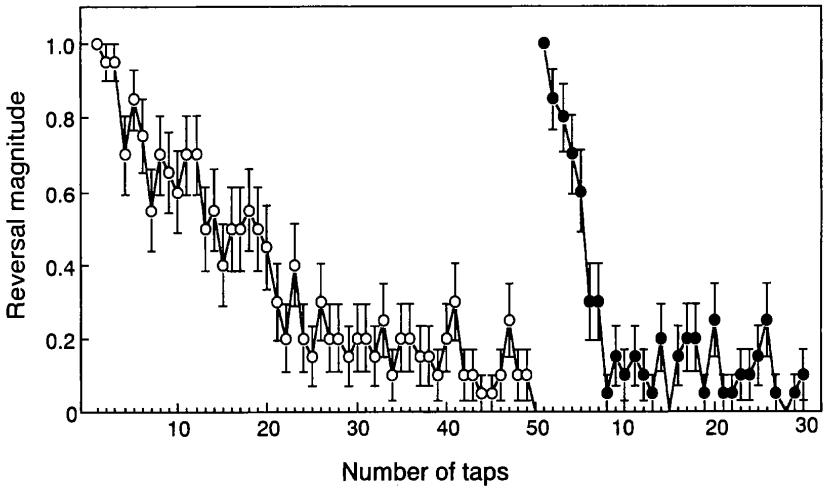

Fig. 4. Retention of habituation. Worms were given 50 serial taps (10-s ISI), during which habituation developed (○). At 5 min after the habituation, the trained worms were given 10-s ISI tap stimuli (●). Mean values (±SE) of responses of 50 worms are presented.

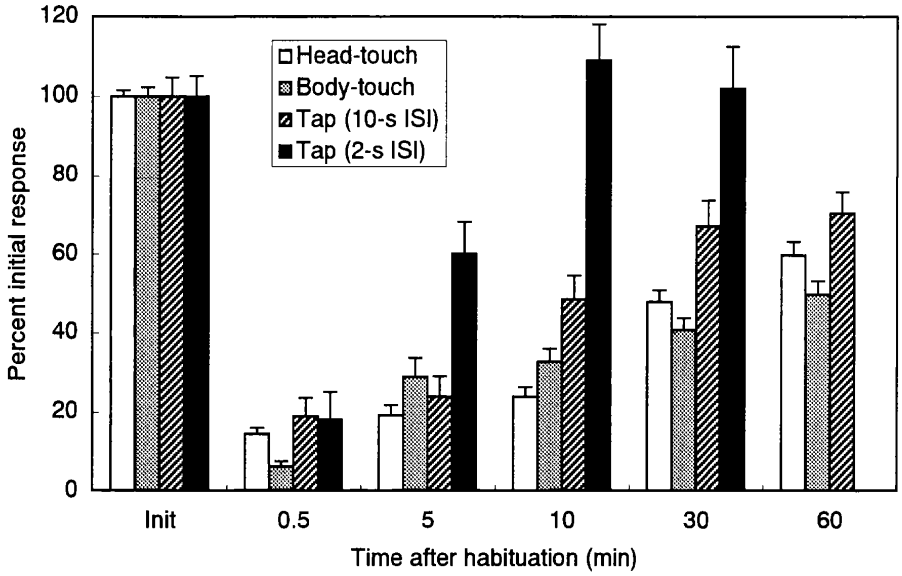

Fig. 5. Relationship between the type of stimuli and the rate of recovery from habituation. Worms were habituated by 50 serial head-touches, body-touches, or taps of 10-s ISI or 2-s ISI. At the indicated times, the recovery from habituation was tested by giving 5 mechanical stimuli. Mean values (±SE) of responses of 50 worms are presented.

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Response of worms under habituation to a different stimulus

Can a worm habituated by one type of mechanical stimulus respond to another type? We tested interrelationships among habituated states evoked by three types of mechanical stimuli (Fig. 6). Worms in a habituated state evoked by 50 head-touches did not respond to body-touch as much as in the initial response (Fig. 6A, left). Conversely, however, worms in the habituated state evoked by 50 body-touches responded to head-touch (Fig. 6A, right). The worms habituated by head-touch or by body-touch responded little to tap, however, these habituated by tap responded to head-touch and body-touch (Fig. 6B, C).

Response of cell-killed worms to touch stimuli

AVAs are interneurons that constitute neural circuitry for backward movement (Fig. 10). The AVA internerons were killed and the touch response was tested. The AVA-killed

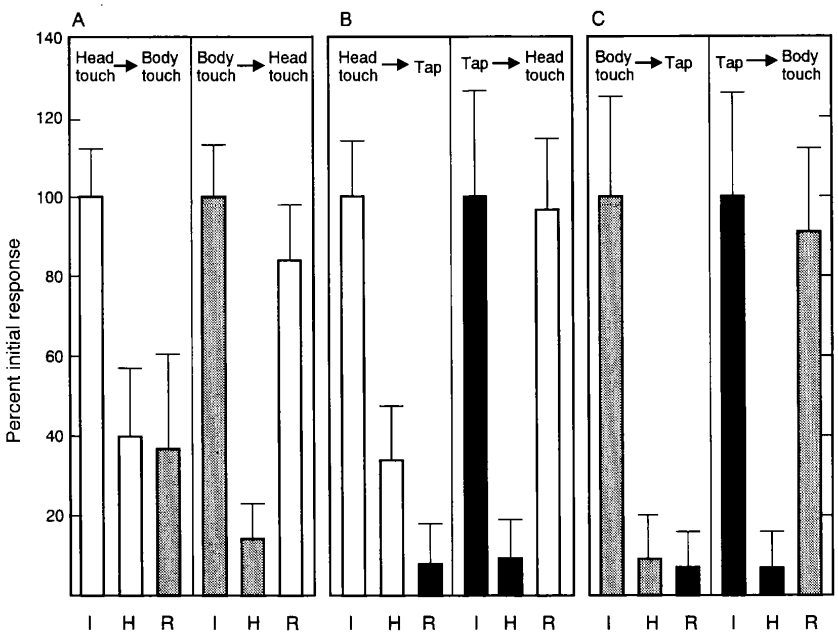

Fig. 6. Initial responses of worms habituated by one type of mechanical stimuli immediately after the habituation by another type of mechanical stimuli. Worms habituated by 50 serial stimuli were tested for their response to another type of stimuli by 5 trials. Each column indicates the mean value (±SD) of the distance of backward movement per body length in 100 trials using 20 worms. I, Initial responses (1~5); H, Habituation responses (46~50); R, Responses to test stimuli (51~55). A: Initial responses to body-touch of worms habituated by head-touch (left), and responses when the stimulus was inverted (right). B: Initial responses to tap of worms habituated by head-touch (left), and responses when the stimulus was inverted (right). C: Initial responses to tap of worms habituated by body-touch (left), and responses when the stimulus was inverted (right).

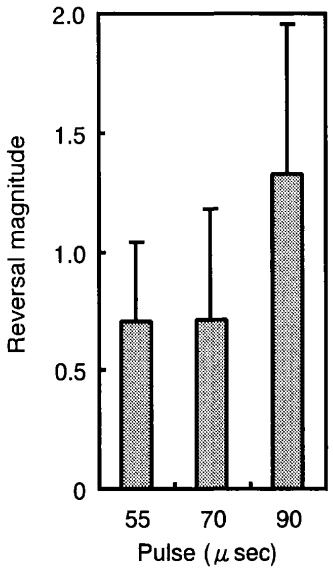

Fig. 7. Response magnitude stimulated by taps of different strengths. Tap stimuli (10-s ISI) with 55, 70 or 90 μsec pulse were given to the worms. Magnitude of response was measured as the distance of backward movement per body length of worms. Each column indicates the mean value (±SD) of 100 trials using 20 worms.

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worms were responsive to touch stimuli, but showed uncoordinated backward movement. Fig. 8 shows the response of the AVA-killed worms under habituation. The treated worms responded more weakly to head-touch or body-touch than control worms, but habituated as the control worms did. The cell-killed or control worms under habituation induced by body-touch responded to head-touch (Fig. 8, top). The worms similarly habituated by head-touch, however, only slightly responded to body-touch (Fig. 8, bottom).

Response of worms to tap stimuli of different strengths

We tested responses of worms stimulated by taps of different strength (Fig. 7). When stimulated by single taps with 55 or 70 μsec pulses, the magnitude of response was not significantly affected ($p=0.9313$). However, taps with 90 μsec evoked significantly ($p<0.0001$) stronger responses than those with 55 or 70 μsec. Fig. 9 shows responses of worms under habituation to one strength of tap to a different strength of tap stimuli. Worms habituated by 50 weaker taps (55 μsec) re-

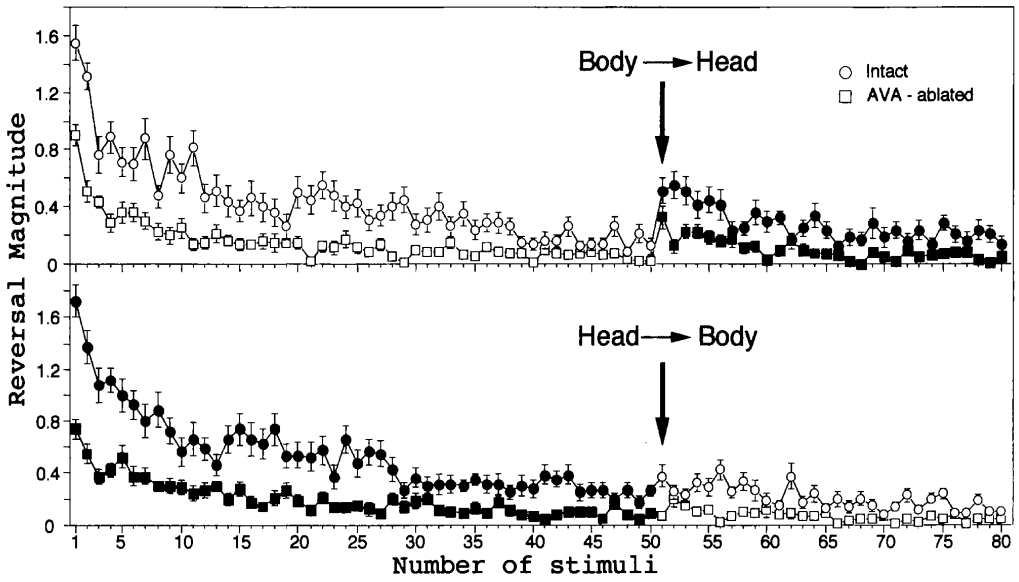

Fig. 8. Response of AVAs-killed worms to either head-touch after the habituation by body-touch (top) or the reversed combination of stimuli (bottom). □, body-touch to AVA-ablated worms; ○, body-touch to intact worms; ■, head-touch to AVA-ablated worms; ●, head-touch to intact worms. Mean values (±SE) of responses of 20 worms are presented.

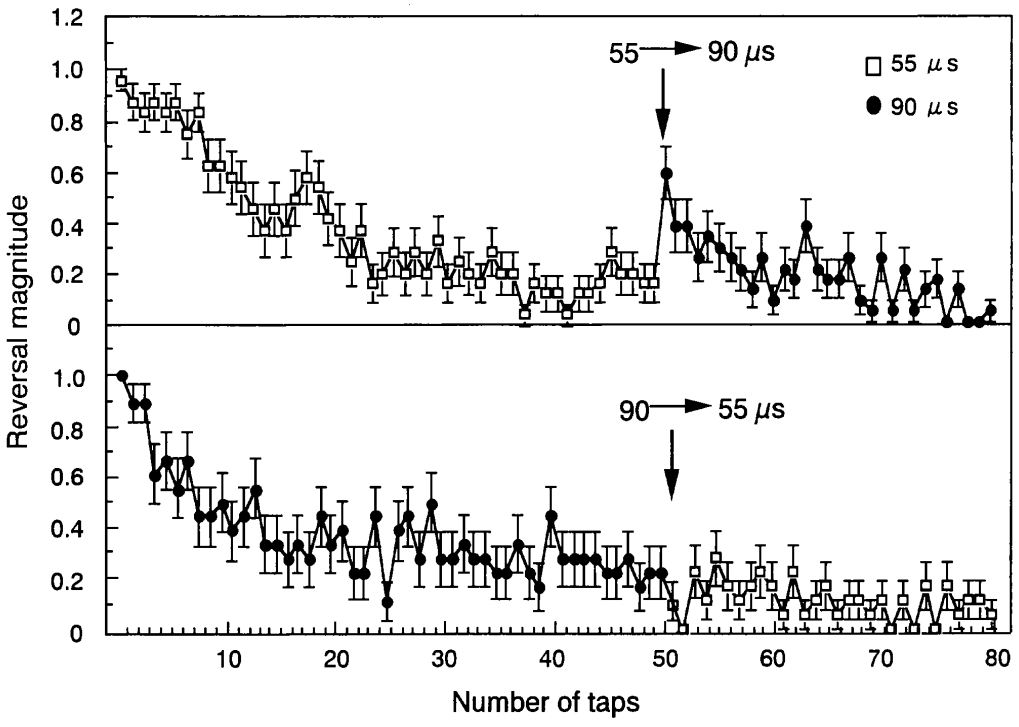

Fig. 9. Effect of strength of stimulus on the habituation. All tap stimuli were given at 10-s ISI. Each point indicates the mean value (±SE) of 20 worms. Top: Worms were habituated by a train of tap stimuli of 55 μsec pulse (1~50), and tested by taps of 90 μsec pulse (51~80). Bottom: Worms were habituated by a train of tap stimuli of 90 μsec pulse (1~50), and tested by taps of 55 μsec pulse (51~80).

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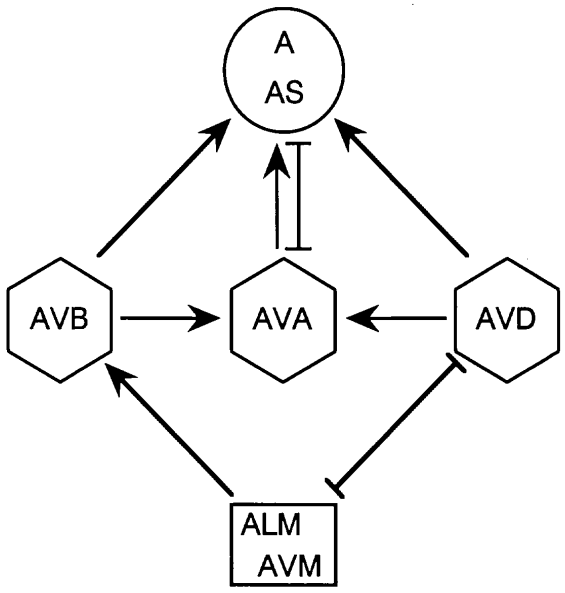

Fig. 10. Simplified neural circuitry mediating backward movement induced by head-touch and body-touch based on results by Chalfie *et al.* (1985) and Wicks *et al.* (1996). □, mechanosensory neuron; ⬡, interneuron; ○, motor neuron; ↑, chemical synapse; I, gap junction.

sponded to yet stronger taps (90 μsec) but habituated much more rapidly than the first time (Fig. 9, top). Worms habituated by 90 μsec-taps did not respond to 55 μsec-taps (Fig. 9, bottom).

DISCUSSION

Habituation of the nematode *C. elegans* was elicited by three types of mechanical stimuli all of which evoked backward movement. We presented results showing that the habituated states of *C. elegans* were not identical, but differed depending on the type of mechanical stimuli given. That is, worms habituated by tap-stimuli were still sensitive to the two types of touch stimuli (on the head and anterior body). However, worms habituated by head-touch were insensitive to tap and body-touch. These results suggest a hierarchy among the habituated states induced by the mechanical stimuli, in the order of head-touch, body-touch, and tap. What is the source of the habituation differences? Two probable causes are the neural circuitry for touch-induced backward movement and the magnitude of stimuli.

The first possibility concerns the touch circuit that integrates mechanical stimuli. All three types of mechanical stimuli given to worms in this study are received and integrated in the neural circuitry for touch-induced movement (Chalfie *et al.*, 1985; Wicks and Rankin, 1995). The touch-circuitry consists of four types of mechanosensory neurons (AVM, ALM, PVM, and PLM), four types of interneurons (AVA, AVB, AVD, and PVC) and two types of motor neurons (A and AS). Touch on the head or on the anterior body stimulates mechanosensory neurons, ALM and/or AVM (Fig. 10). The stimuli are then transmitted to interneurons which integrate the stimuli and transmit the information either into the A motor neurons or directly to the AS motor neurons, and evoke a withdrawal reflex. Tapping, however, stimulates not only ALM and/or AVM but also PLM. The information transmitted from these three types of mechanosensory neurons are integrated by the interneurons and evoke backward movement (Wicks and Rankin, 1995). Therefore, the hierarchy may be caused by topological differences in the neural circuitry receiving the mechanical stimuli. However, another sensory neurons such as ASH may be stimulated by the head touch.

Another possibility infers that the strength of stimuli causes the hierarchical difference in the habituated states of worms. The strength of stimuli is presumably different among the three mechanical stimuli because the magnitude of the response of worms is different. The order of magnitude of response (that is, the supposed strength of stimuli) is touch at the anterior region and tap (Fig. 2). In addition, worms habituated by weaker taps responded to stronger taps (Fig. 9). These results imply that, in the hierarchy, the strength of the stimulus is more important than the neural circuitry. The habituation tests with AVA neuron ablated worms indirectly support this idea (Fig. 8). Worms with ablated interneurons still habituated when given touch stimuli at the head or anterior body, indicating that intact neural circuitry mediating the backward response is not essential for habituation. The hierarchical relationships of habituation induced by the head touch and the anterior body touch between AVA-ablated and the intact worms were essentially identical. Both stimuli are presumably transmitted through AVB, AVD and AVA in the intact worms, while in the AVA-ablated worms, stimuli are presumably transmitted through AVB and AVD. Therfore, the hierarchy is not likely induced by a specific neural pathway. It is not yet known how the difference of strength of stimulus is integrated in the touch circuit. To elucidate which possibility is the case, molecular and genetic analyses of the habituation of *C. elegans* will be indispensable.

In the marine snail *Aplysia*, habituation of the gill withdrawal reflex results from the defects of synaptic transmission from the sensory neurons (Hawkins *et al.*, 1983; Fisher *et al.*, 1997). In *C. elegans*, the loci of change associated with habituation of the tap withdrawal reflex are also supposed to be at the presynaptic terminals of sensory neurons (Wicks and Rankin, 1997). Do identical neurons contribute to the generation of habituated state and its maintenance in the habituation produced by mechanical stimuli? To test the role of each neuron in the touch-circuit for habituation, laser ablation of these cells may hold promise (Wicks and Rankin, 1996b). The molecular mechanisms of habituation are another area in this field that awaits study. As such, genetic analyses of habituation defective mutants are in progress in our laboratory. We have isolated several such mutants of *C. elegans*, which we hope will serve to solve the problems of learning and memory outlined in this paper.

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