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# Memory in Fruit Flies and Nematodes

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A change in the behaviour of an organism that results from that individual's exposure to stimuli/events in the environment. *Drosophila melanogaster* and *Caenorhabditis elegans* have been the focus of much research in the areas of learning and memory.

## Introduction

Learning and memory are expressed as changes in behaviour as a result of experience. Learning is an essential part of existence for organisms ranging from one-celled protozoans to humans. Considering the importance and apparent universal properties of simple forms of learning, such as habituation, sensitization and classical conditioning, these processes probably evolved early in evolution, and their basic cellular mechanisms may have been highly conserved. This view has led researchers to study the mechanisms of these forms of learning and memory in simple systems, hoping to extrapolate their results to more complex organisms.

Learning is a change in behaviour dependent only on an organism's experience; it is not the result of sensory adaptation, fatigue, injury or drugs. The most common distinction made in learning theory is between nonassociative and associative forms. Nonassociative learning (i.e. habituation or sensitization) is the result of experience with a single stimulus (delivered once or repeatedly). Associative learning occurs when an organism makes a connection between two stimuli, as in classical or operant conditioning. Memory involves the storage or maintenance of this learning.

The fruit fly, *Drosophila melanogaster*, and the nematode, *Caenorhabditis elegans*, have become the focus of much research in the area of learning and memory. They are excellent examples of how a simple-system, multi-disciplinary approach can greatly advance our understanding of complex behaviours. Not only do we gain a great deal of knowledge on the behaviour being studied, but techniques are developed that can then be modified for use on other, more complex organisms.

## Genetic Manipulation of Fruit Flies and Nematodes

Researchers studying *D. melanogaster* and *C. elegans* have at their disposal powerful genetic techniques with which to

examine learning and memory at a basic level. Both these organisms possess traits necessary for genetic studies: they are easy to culture, prolific and have short life spans (Tully, 1991).

*D. melanogaster* possesses a small genome,  $1.65 \times 10^8$  base pairs on five chromosomes (three autosomal, one X and one Y), which makes it an attractive organism for genetic analysis. It is capable of both associative and nonassociative forms of learning, allowing researchers to study these behaviours at a genetic level.

*C. elegans* also has a small genome, consisting of  $8 \times 10^7$  base pairs on five autosomes and one sex chromosome, which has been almost entirely mapped and sequenced. *C. elegans* are self-fertilizing hermaphrodites, allowing for maintenance of homozygous strains. However, males can arise spontaneously through nondisjunction of the sex chromosome and can be used to create heterozygous balancer strains. *C. elegans* may be frozen at  $-72^\circ\text{C}$  indefinitely, enabling a library of mutants to be kept, until a specific mutant is required for study, when the frozen worms may be thawed and allowed to reproduce.

## Mutagenesis and behavioural screening

The basic strategy for using genetics to study learning and memory is the same for both organisms: develop an assay for the desired behaviour (Table 1) and then screen a mutagenized population of animals or a known mutant for abnormalities in that behaviour. Although mutations occur spontaneously, the rate of this is too infrequent for it to be useful. Many methods exist to produce mutants, including exposure to X-ray radiation and chemical mutagens. The most common method used in the work discussed here is the chemical mutagen ethylmethanesulfonate (EMS). EMS is a potent mutagen that produces point mutations (changes in a single base pair of DNA). Once an organism is mutagenized, it is allowed to reproduce. True breeding populations are maintained for fruit flies; worms are only allowed to self-reproduce. The

progeny are then screened in the behavioural assays. Once an abnormality is found for the behaviour of interest, the mutant must then be analysed to ensure not only that it is a single gene mutation but also that it is a true learning and memory mutant. This necessitates that the abnormal behaviour is not due to a confounding deficit (i.e. sensory or motor impairment), or developmental deficits that lead to morphological changes in structure. Any of these factors may produce abnormal performance that is not due to a specific learning and memory impairment. Once a mutation that affects learning and memory has been isolated, it is then possible to identify the affected gene and determine its role and method of action.

## Transgenes

A relatively new tool for the analysis of the genes underlying learning and memory is reverse genetics using transgenes. Once a gene has been identified, cloned and sequenced, the DNA can be manipulated and transgenes made. Transgenes are segments of cloned DNA containing the control (promoter) and coding elements of a gene. The construction of transgenes involves using techniques to isolate and 'cut and paste' sequences of DNA, allowing the control section of one gene to be placed in front of another so that expression of the gene of interest can be controlled. These segments of DNA are injected into oocytes or embryos, where the transgene can be incorporated into an organism's DNA (in *D. melanogaster*) or can remain extrachromosomal (in *C. elegans*). Transgenes can be used to: insert a blocker or null of a gene, creating a loss or reduction of function; add extra copies or different alleles of a gene, causing a change or gain of function; rescue a mutant phenotype by replacing the affected gene; or attach reporters or promoters to the desired gene to see its morphological expression patterns. All of these techniques add to the ability to investigate the role of a particular gene in a given behaviour.

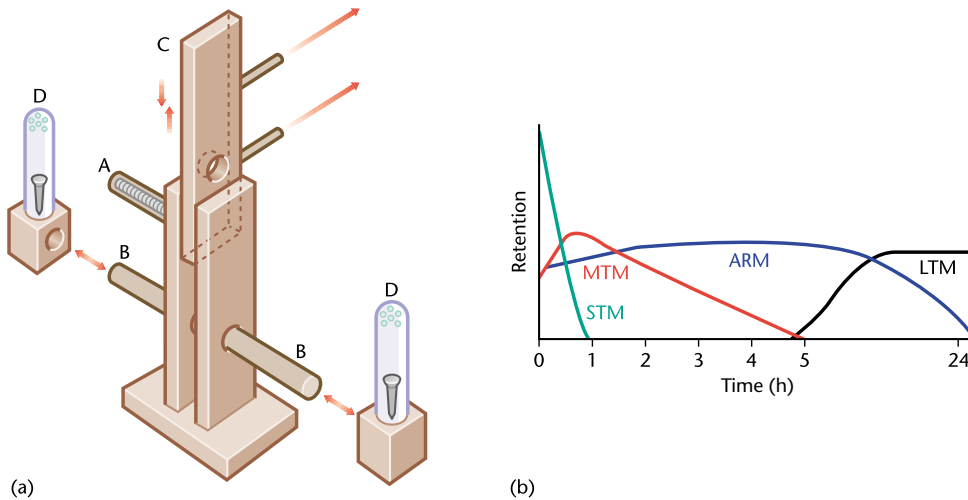
Some exciting transgene work employs the use of reporter genes and promoter sequences. Reporter genes such as *lacZ* and green fluorescent protein (gfp) are attached to the promoter sequence of an identified gene and can then be visualized in any cell where the gene is activated. This allows the identification of the cells in which the gene is turned on and the determination of when during ontogeny a gene is active. One of the problems with studies of specific genes for learning and memory is that the genes may also play a role in a variety of aspects of cell development and normal physiology. Thus, distinguishing pleiotropic or developmental abnormalities caused by a gene from specific effects on learning and memory is difficult. Two methods have been developed to address this problem – one restricts the expression of transgenes to only a few cells, the other restricts the temporal expression of transgenes. Spatial control over gene expression has been

achieved using mosaic analysis and enhancer-trap systems. Mosaic analysis involves generating an organism in which most cells develop normally and only some cells are genotypically mutated by using transgenes with promoters that activate a gene in only a specific subset of cells. In *D. melanogaster*, enhancer-trap techniques target gene expression or disruption to specific regions via tissue-specific enhancers of gene transcription (DeZazzo and Tully, 1995). Temporal control over gene expression is accomplished with special types of promoters that control when a gene is turned on, so that an organism may develop normally and then, once it becomes an adult, gene expression can be disrupted. For example, in both *C. elegans* and *D. melanogaster*, heat shock promoters (HSPs) allow us to turn on the gene only when we apply heat shock to the organism or cell in question (DeZazzo and Tully, 1995). Thus the organism develops normally and has its genetic expression pattern altered only after it is fully formed; this eliminates possible developmental confounds. Recent experiments have tried to combine tissue specific expression with temporal control of expression so that genes can be altered only when and where the researcher desires.

## Fruit Fly Conditioning Paradigms

The first step in the investigation of the genetic basis of learning and memory in any organism is to develop paradigms in which the organism displays one or more forms of learning. In *D. melanogaster*, a number of paradigms have been developed to study both associative and nonassociative learning (for a detailed review of *D. melanogaster* learning assays see Davis, 1996). Nonassociative assays involve presentations of a single stimulus. Two major forms of nonassociative learning that occur are sensitization and habituation. Sensitization is an increase in response to a noxious stimulus, while habituation is a decrease in response to repeated presentations of a stimulus. The proboscis extension reflex is one of the more common reflexes studied in both sensitization and habituation paradigms in *D. melanogaster*. A sucrose solution is applied to the proboscis of the fly. When a concentrated sucrose solution is used, the animal becomes sensitized and will even display the reflex in response to water (which does not normally occur). However, when a weak sucrose solution is used, the animal habituates and the reflex weakens. Other nonassociative paradigms include acoustic sensitization, habituation of the landing response, and habituation and sensitization of the cleaning reflex.

Associative learning paradigms are usually divided into two types: classical and operant. In classical conditioning, two stimuli are paired temporally so that one stimulus becomes the predictor of the other, while in operant conditioning the probability of a response occurring is



**Figure 1** (a) Apparatus used to measure classical conditioning in *D. melanogaster*. Training phase: groups of flies (~150) are placed into the training tube (A), which is lined with an electrifiable grid. Vacuum-generated air currents are used to deliver the concentrated sucrose CS+ and CS- odours (D) to the training tube, one at a time. The CS+ odour is presented with a shock, while the CS- is not. Testing phase: the flies are then moved down to the testing choice point, via an elevator (C). During testing, the flies have the choice of moving into one of two test tubes (B); one contains the CS+ odour and the other the CS- odour. The tubes are then removed and the number of flies in each is counted to assess whether learning (to avoid the odour paired with the shock) has occurred. (Adapted from Tully (1991).) (b) Putative stages of memory in *D. melanogaster*. The four stages: short-term memory (STM), middle-term memory (MTM), anaesthesia-resistant memory (ARM), and long-term memory (LTM) are functionally independent and act additively to produce the observed learning curves. (Modified from DeZazzo and Tully (1995).)

determined by the outcome (reward or punishment) following the previous response. The learning paradigm that was utilized in the majority of the work discussed here is a form of classical conditioning called olfactory shock-avoidance conditioning. In the training phase, flies are placed in a test tube (Figure 1a for apparatus). When the first odorant is puffed into the test tube, the flies receive a shock from a wire that surrounds the test tube – the odorant and the shock are paired. When the next odorant is puffed into the test tube the flies receive no shock. Exposure to two odorants and the shock forms one training trial. In memory studies flies receive a number of training trials; these trials can be presented all at once without a break (massed training), or they can be spaced over time with breaks of 10–60 min between trials (distributed training). In the testing phase, the flies are given a choice of the two odorants and 85% of them avoid the odorant that was previously paired with shock. This indicates that the flies have learned the association between the odorant and the shock. This memory can last for several weeks. Other associative learning paradigms used to study *D. melanogaster* include sucrose reward learning, colour discrimination, conditioned courtship with mature females, leg position conditioning, and flight orientation learning.

Using these paradigms, a number of different mutants have been isolated which have different effects when tested in different behavioural paradigms. Not all mutant strains have been tested in all paradigms.

## Fruit Fly Learning Mutants and Their Biochemical Defects

See Table 1.

## Molecular Genetic Mechanisms of Learning and Memory

Using the olfactory shock avoidance paradigm, a number of single-gene *D. melanogaster* mutants with deficits in learning and memory that involve the cyclic adenosine monophosphate (cAMP) cascade have been isolated. The intracellular cAMP metabolic cascade has been the focus of much attention, and is thought to play a key role in the processes underlying learning and memory. Work by Kandel on the marine mollusc *Aplysia californica* provided much of the early physiological evidence for the role of the cAMP cascade in learning and memory. Work in *D. melanogaster* has also contributed evidence that supports the role of the cAMP cascade: among the cAMP cascade mutants are *dunce* (phosphodiesterase), *rutabaga* (adenylylate cyclase) and *DCO* (protein kinase A, PKA). Although the finding of these mutants does much to support the role of the cAMP cascade in learning and memory, it does not imply that this is the only mechanism. A close look at

**Table 1** Learning and memory mutants (without identifiable brain structure defects) in *D. melanogaster*<sup>a</sup>

Mutant	Preferential site of gene expression	Biochemical defect	Associative learning	Nonassociative learning	Memory	Other behavioural defects
<i>dunce</i>	Mushroom bodies	Deficient in cAMP phosphodiesterase	Poor	Poor sensitization; poor habituation (PER); faster habituation (LR)	Impaired STM	Sterility in females
<i>rutabaga</i>	Mushroom bodies	Deficient in calcium/calmodulin-activated adenylyl cyclase	Poor	Poor sensitization; poor habituation (PER); faster habituation (LR)	Impaired STM	Suppression of female sterility in <i>dunce</i>
<i>DCO</i>	Mushroom bodies	Deficient in PKA	Poor		Impaired STM and MTM	Lethality
<i>Shaker</i>	Mushroom bodies	Defective voltage-gated potassium channels	Poor		Impaired STM	
<i>Ether-a-go-go</i>		Defective voltage-gated potassium channels	Poor			
<i>Amnesiac</i>		Deficient in pituitary adenylyl cyclase activating peptide	Poor	Poor sensitization; poor habituation (PER); faster habituation (LR)	Impaired MTM	Suppression of female sterility in <i>dunce</i>
<i>Radish</i>		?	Poor		Impaired ARM	
<i>Cabbage</i>		?	Poor			
<i>Turnip</i>		Defective PKC activation	Poor	Poor habituation (PER)	Impaired MTM	
Dopa decarboxylase		Defective biosynthesis of dopamine and serotonin	Poor			Lethality
Protein phosphatase-1 ( <i>Su-var(3) 6</i> )		Deficient in protein phosphatase-1 activity	Poor	Poor sensitization; faster habituation (LR)	Impaired STM	Lethality

*continued*

Table 1 – continued

Mutant	Preferential site of gene expression	Biochemical defect	Associative learning	Nonassociative learning	Memory	Other behavioural defects
Calcium/calmodulin-dependent protein kinase (CaM kinase II)		Deficient in CaM kinase II	Poor	Poor sensitization	Poor	
CREB		Deficient CREB	Poor		Impaired LTM	
<i>Volado</i>	Mushroom bodies	Deficient in $\alpha$ -integrin molecule			Impaired STM	

CREB, cAMP response element-binding protein; LR, landing response; PER, proboscis extension reflex; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C.  
 \*Adapted from Davis (1996).

Table 1 shows that defects in ion channels, neurotransmitter production, and protein phosphatase activity also affect learning and/or memory.

A four stage theory of memory has been proposed in *D. melanogaster* (DeZazzo and Tully, 1995). The evidence for these phases is based on comparing retention curves for olfactory conditioning from wild-type and mutant flies. Mutations were isolated that caused deficits in performance when tested at particular time points after training. The putative memory stages begin with acquisition, in which mutants that are defective in learning perform poorly. Following acquisition, information is passed sequentially through short-term memory (STM; minutes to hours) and by middle-term memory (MTM; hours to a day), which are functionally distinct. It is then consolidated into two long-lasting memory phases which act in parallel: anaesthesia-resistant memory (ARM; about 1 week) and long-term memory (LTM; several weeks). The phases are thought to act additively to produce the observed retention curve (Table 1 and Figure 1b).

Although ARM and LTM are both considered longer-lasting stages, it is LTM that constitutes 'permanent' memory. LTM requires protein synthesis and is disrupted by procedures or drugs (i.e. cold temperatures or anaesthetics) that interfere with protein synthesis. LTM is produced only by distributed training procedures. In contrast, ARM is resistant to protein synthesis inhibitors and cold temperature anaesthesia. It is produced by both massed and distributed training procedures. The long-lasting effects of both ARM and LTM seem to involve the catalytic subunit of PKA. The difference appears to be that in ARM the PKA subunit acts cytoplasmically, while in LTM it translocates from the cytoplasm to the nucleus (Tully, 1991).

One aspect of LTM that has received much attention and investigation is the role of the gene for cAMP response element-binding protein (CREB) on LTM consolidation. Following distributed training, the catalytic subunit of PKA translocates from the cytoplasm into the nucleus of the neuron. Once in the nucleus it phosphorylates CREB, thus activating it. CREB activation causes the transcription of immediate early genes, which in turn can then, with CREB, cause the transcription of late response genes. These late response genes appear to encode proteins necessary for long-term synaptic change (Carew, 1996). Studies in *D. melanogaster*, *A. californica* and mice with CREB transgenes have shown that LTM is enhanced with increased CREB activation. In addition, blockers of the CREB gene (or an inhibitory CREB gene) have been shown to block LTM consolidation. CREB is an excellent example of the conservation of genes controlling learning and memory across species.

Genetic techniques can also be used to determine areas of the brain that might be crucial for learning and memory. This can be accomplished by screening for mutant strains that have morphological defects in different brain struc-

tures. By doing these types of study, researchers have suggested that specific regions of the mushroom bodies in the flies' brains are the site of the classical conditioning seen in olfactory shock avoidance conditioning.

## Sex and Learning in Fruit Flies

Another paradigm used in *D. melanogaster* examines the role of learning and memory in male mating behaviour. When they become sexually mature, males will attempt to copulate with already fertilized females, virgin females and immature males. If a male attempts to copulate with an already fertilized female, he will be rejected. Courtship attempts will decrease in intensity and frequency after extended contact (~60 min) with unreceptive flies. This decline in copulation attempts has been shown to enhance short-term reproductive fitness (Hall, 1994). The results of a number of studies have shown that male flies associate the odour released by the fertilized female with lack of receptivity. Experienced wild-type flies spend less time courting when in the presence of the odour of fertilized females.

This decline in courtship after pairings with mated females requires that the males learn and remember the association between an odour and decreased reproductive success. Several of the mutations that express a deficit in associative learning in the odour shock avoidance paradigm also express a deficit in courtship conditioning. *Amnesiac* exhibits depressed courtship for only about 30 min; *dunce*, *rutabaga*, *shaker* and *ether-a-go-go* also show deficits in this conditioned decrease in courtship.

Female flies' responsiveness to courtship can also be altered by experience. In this case, it is an increase in response resembling behavioural sensitization. If females are exposed to electronically simulated courtship songs, they show a decreased latency to allow mating when placed with males (Kyriacou and Hall, 1994). This enhanced receptivity lasts approximately 1–3 min after exposure to the song. Examples of mutations that ablate this enhanced receptivity are *dunce*, *rutabaga* and *amnesiac*.

Experience-dependent courtship is an example of the adaptive advantage that the ability to learn presents. Courtship and copulation represent an energy cost to the male. If males can learn and remember where it is appropriate to expend energy, and where it is not, they can spend their reproductive hours courting females that are most likely to pass the male's genes to offspring.

## Learning in Nematodes

The study of learning in *C. elegans* benefits from a variety of disciplines, including biochemistry, genetics, molecular biology, neurobiology, physiology and psychology. *C.*

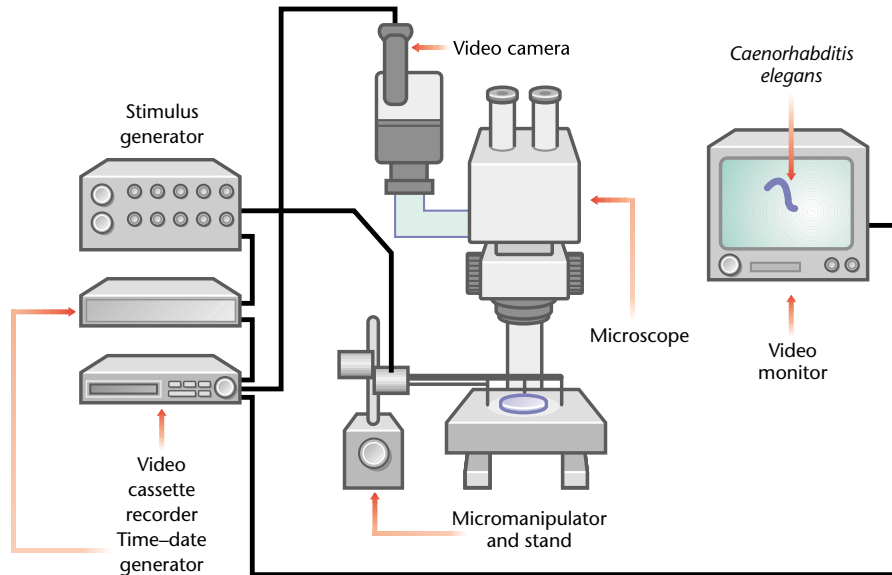
*elegans* has a simple nervous system consisting of 302 neurons, all of which have a known lineage and connectivity. Its genome, as previously discussed, is relatively small and has been extensively studied. *C. elegans* is transparent, which allows the use of laser ablation to kill individual cells in a live animal. This transparency also allows the visualization of reporter genes in an intact, living worm.

The first paradigm used to demonstrate learning in *C. elegans* was the tap withdrawal response first described by Rankin *et al.* (1990). This protocol involves administering a series of mechanical taps to the side of the Petri dish containing the animal to assess habituation. The apparatus used for these studies is shown in **Figure 2**. In *C. elegans*, repeated taps lead to habituation of the worm's response to this stimulus, which is to swim backwards. Habituation is seen as a decrease in both the length and frequency of the worm's reversals. The degree to which worms both habituate and recover from habituation is sensitive to the rate at which stimuli are delivered; worms habituate and recover more rapidly to shorter interstimulus intervals than to longer ones.

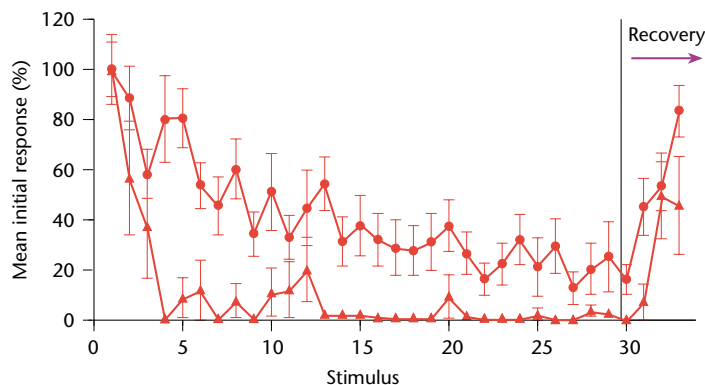
Worms are also capable of long-term memory for habituation training. In this paradigm, worms are given three blocks of 20 stimuli at 60-s intervals, with an hour's rest between blocks; they were tested 24 h later with a block of 20 stimuli at 60-s intervals. The results show that worms are capable of remembering habituation training for at least 24 h. Long-term memory can be disrupted by heat shock during the rest intervals. The current hypothesis is that heat shock is disrupting cellular processes, such as protein synthesis, that are necessary for memory formation.

Studies investigating the neural circuit underlying the behaviour show that two opposing neural circuits are activated by the tap: tail touch receptors activate interneurons that direct forward movement; and head touch receptors activate interneurons that direct backward movement. Interestingly, the two circuits have different patterns of behavioural output in response to habituation training (Wicks and Rankin, 1996), again suggesting that habituation in intact animals is the result of a balance of two competing behaviours – reversals and accelerations. These results suggest that there might not be a single mechanism underlying habituation, nor might all cells involved in a behaviour respond in the same way to repeated stimulation, but rather each cell or cell type may have a unique response to repeated stimulation, and the behaviour that is observed is the integrated output of all of the cell types.

The first mutant strain to show a deficit in habituation is the *eat-4* (*ky5 III*) mutation. The *eat-4* mutant is defective in feeding, sensitivity to nose touch, chemotaxis and thermotaxis. Using both *lacZ* and *gfp*, Lee *et al.* (1995) as cited in Peters *et al.* (in press) demonstrated that *eat-4* is expressed in a number of pharyngeal neurons in addition to



**Figure 2** Apparatus used to assess habituation in *C. elegans*. Individual worms are placed on to a Petri dish, held in a plastic attachment that is connected to a micromanipulator. A mechanical taper driven by an electromagnetic relay is used to deliver the taps in a consistent and uniform manner to the Petri dish from a Grass S88 stimulator. A video camera attached to the microscope and connected to a video monitor and video cassette recorder is used to monitor and record the behaviour of the worm. To allow for precision of the timing of events, a time-date generator is used to superimpose the time (in thousands of a second) and date of the experiment on to both the monitor and the recorder. After habituation training, the magnitude of the responses to tap are quantified using stop-frame video analysis by tracing each response on to an acetate, which is then scanned into a microcomputer to measure the length of each response. (Adapted from Peters *et al.* (in press)).



**Figure 3** Habituation curves for the tap-withdrawal response in (solid circle) wild-type (N2) and (solid triangle) *eat-4* strains of *C. elegans* at a 10-s interstimulus interval. Habituation is assessed by measuring the distance the worms travel backwards in response to tap. *Eat-4* display an accelerated rate of habituation and slower recovery from habituation training than do wild-type worms. (Adapted from Peters *et al.* (in press).)

mechanosensory neurons and suggested that *eat-4* is responsible for glutamergic transmission in these cells. *Eat-4* is hypothesized to be a glutamate transporter involved in regulating glutamate in terminals. In tests of habituation, *eat-4* exhibits a number of features that may allow it to be considered a 'learning' mutant. For example, *eat-4* worms demonstrate an accelerated rate of habituation, a depressed asymptotic level of habituation, and a rather retarded level of recovery from habituation when compared with N2 wild-type worms (Figure 3). Thus, *eat-4*

worms may be characterized as 'fast' learners and appear to retain such learning for longer periods of time than normal worms.

*C. elegans* is also capable of demonstrating associative learning. For example, *C. elegans* can learn to associate food with a particular chemoattractant. Wen *et al.* (1997) exposed animals to either sodium or chloride ions, one of which was associated with the presence of food (*Escherichia coli*), the other with the absence of food. Later, when placed on to a plate with both ions available, the animals

migrated towards the ion that was paired with the food. In addition, the worms demonstrated conditioned aversion to an ion (sodium or chloride) that had been previously paired with garlic. Therefore, it appears as though *C. elegans* can be conditioned to either approach or avoid ions on the basis of past experience. Using this protocol, van der Kooy et al. have isolated two mutant strains, *lrn-1* and *lrn-2*, that do not appear to show associative learning (Wen et al., 1997). The mutations involved in *lrn-1* and *lrn-2* have been mapped to chromosomes; however, the gene products have not yet been identified.

## Summary

Research on the genetics of learning and memory in *D. melanogaster* has contributed to our understanding of the biochemistry of learning and memory and the organization of memory itself. New work on localization of specific defects to specific brain centres is adding to our understanding of the organization of nervous systems and the role these areas play in learning and memory. Research on *C. elegans* has demonstrated that this simple worm can learn, and, with the discovery of mutants that show abnormal habituation and mutants that show abnormal classical conditioning, it is apparent that this organism will be a useful tool for the genetic analysis of learning and memory.

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