avr-14 Selectively Affects Short-term and Long-term Habituation at Short Interstimulus Intervals in *Caenorhabditis elegans*

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Abstract

The role of interstimulus interval (ISI) in habituation has been well documented in a large number of species. Habituation occurs at a faster rate when stimuli are presented at a high frequency with short ISIs and at a slower rate when stimuli are presented at a low frequency with long ISIs. Spontaneous recovery, a return of response amplitudes to pre-habituation levels upon cessation of stimulation, is also affected by ISI, such that spontaneous recovery occurs more rapidly when trained with short ISIs and less rapidly when trained with long ISIs. This has led to the hypothesis that short-term habituation is mediated by a family of ISI-sensitive cellular mechanisms that somehow encode the ISI of training and set the rate of spontaneous recovery. If this is the case, then there should exist genetic mutations that selectively affect habituation at short ISIs, long ISIs, or more than one ISI. The gene *avr-14* encodes for a glutamate-gated chloride channel expressed on the sensory neurons of the tap-withdrawal (TWR) circuit in *Caenorhabditis elegans*. Results show that mutations in *avr-14* affect both short-term and long-term memory in an ISI-dependant manner. Short-term habituation at short ISIs (10s and 30s) was affected while short-term habituation at long ISIs (45s and 60s) was not. Long-term memory for distributed or massed training at a 60s ISI did not appear to be affected by mutations in *avr-14*. On the other hand, worms with mutations in *avr-14*, unlike wild-type worms, showed significant long-term memory for distributed training with a 10s ISI. This form of memory was shown to be dependent on protein synthesis. Results from the series of experiments in this paper support the hypothesis of multiple short-term memory systems. The effect of mutations in *avr-14* on long-term memory are very unique as there exists no other example in the literature of a gene whose presence blocks the formation of long-term memory.

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avr-14 Selectively Affects Short-term and Long-term Habituation at Short Interstimulus Intervals in *Caenorhabditis elegans*

Habituation is defined as a decrement in responding following repeated exposure to the same stimulus that is not the result of receptor adaptation or fatigue (Thompson & Spencer, 1966). This simple, non-associative form of learning is conserved across evolution, and has similar characteristics throughout the animal kingdom. The ubiquity of habituation is suggestive of its adaptive significance.

An animal will habituate if the repeated stimulus has no obvious biological significance. In other words, if nothing important follows the stimulus, then with repeated exposure the animal will show habituation. Continuing to respond to a stimulus that carries little or no significance may quickly become costly to the animal. The fact that animals show weaker and less rapid habituation to strong stimuli (Thompson & Spencer, 1966) supports this notion. A strong stimulus is more likely to be associated with something threatening to the animal and thus more likely to carry potential biological significance. That habituation is weaker and takes longer to occur in such a situation seems reasonable.

It has been suggested that habituation forms the basis of selective attention and thus the basis for all other forms of learning (Steidl & Rankin, in press). If animals or humans did not habituate they would never get past the point of paying equal attention to everything in their environments. This would leave them incapable of selectively attending to events that are biologically significant, such as those predictive of danger or food.

A seminal paper published more than thirty years ago (Groves & Thompson, 1970) outlined basic characteristics of habituation for all organisms. Across the large number of species that have been studied to date, habituation follows the same unique pattern, reflected in characteristic response curves observed during habituation training. Animals will respond

to the first few stimuli after which habituation develops exponentially until reaching asymptotic levels. The two components are reflected as a sharp drop in the response curve and essentially a flattening of the curve with no further response decrements. When stimulation stops responses will recover to pre-habituation levels, a process referred to as spontaneous recovery (Thompson & Spencer, 1966). Another hallmark of habituation, that sets habituation apart from fatigue, is the presence of dishabituation such that when a habituated organism is presented with a novel or strong stimulus its response levels are restored to pre-habituation baseline levels.

There is an inverse relationship between stimulation frequency and habituation rate such that habituation consistently occurs more rapidly when stimuli are presented at a high frequency with short interstimulus intervals (ISIs) than at a low frequency with long ISIs. Recently it has been shown that spontaneous recovery is affected by the ISI used during training as well, such that spontaneous recovery occurs more rapidly when trained with short ISIs and less rapidly when trained with long ISIs (Rankin & Broster, 1992). The effect of ISI on the rate of spontaneous recovery can also be used to distinguish habituation from fatigue.

Even very simple organisms such as the single-celled protozoan *Stentor coeruleus* shows stereotypic habituation of a contractile response following repeated mechanical stimulation (Wood, 1970). A variety of invertebrate species show similar behavioural patterns. Examples include habituation of movement responses to both light onset and water current in the leech (Ratner, 1972) and habituation of the gill-withdrawal reflex in response to tactile stimulation of the siphon in *Aplysia californica* (Pinsker, Kupferman, Castellucci, & Kandel, 1970). There are examples of stereotypic habituation in vertebrate species coming from both reduced preparations (Thompson & Spencer, 1966; Farel, Glanzman, & Thompson, 1973) and intact animals (Davis, 1970). Thompson and Spencer (1966) gathered data from a

spinal cat preparation, while Farel and colleagues (1973) used a similar approach in isolated frog spinal neurons. Davis (1970) documented the ISI effect on habituation of the startle response in rats. Finally, an example of the characteristic ISI effect on habituation in humans comes from an experiment involving analysis of the orienting response, using the galvanic skin response as the measure (Geer, 1966).

Although habituation is commonly referred to as a "simple" form of learning, surprisingly little is known about the neural mechanisms that mediate this process. Attempts at understanding the molecular mechanisms and the genetic bases of habituation have greatly benefited from a reductionist approach. More broadly speaking, the study of the synaptic plasticity underlying learning and memory in general, has gained much from this approach. By using organisms with simpler nervous systems amenable to cellular and genetic analyses (*C. elegans*, *Drosophila*, *Aplysia*) the number of potentially confounding variables is greatly reduced allowing researchers to ascribe with more confidence any observed changes to specific experimental manipulations. Overall, determining the neural and cellular mechanisms underlying habituation has proved elusive. Research using *Aplysia californica* has suggested that habituation may involve a decrease in available neurotransmitter due to a depletion of the number of vesicles immediately adjacent to the active zone of the sensory neuron (Castellucci & Kandel, 1974; Bailey & Chen, 1988), combined with a decrease in calcium current (Klein, Shapiro, & Kandel, 1988) in the sensory neuron. The role of calcium in the mechanism(s) underlying habituation is not clearly understood. More recent work in cultured *Aplysia* neurons has provided evidence against the hypothesis that habituation results from decreases in presynaptic calcium influx (Armitage & Siegelbaum, 1998). Measuring the presynaptic calcium transient showed no change, while simultaneous recording of postsynaptic EPSPs showed progressive depression of EPSP amplitude with repeated presynaptic stimulation, as is

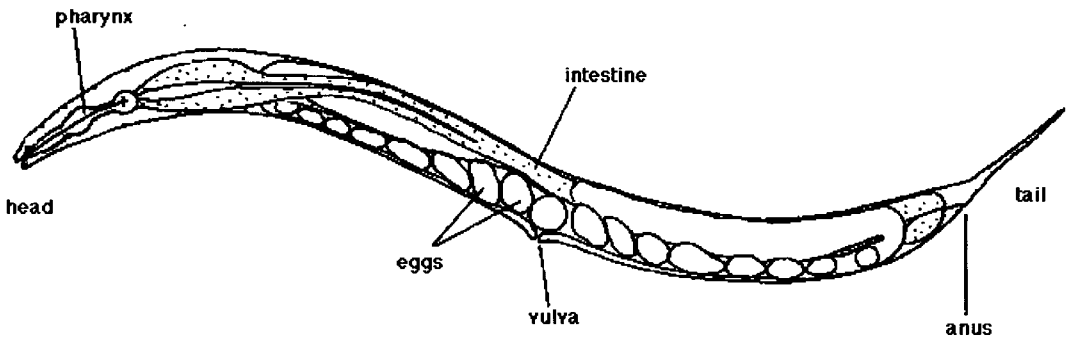
characteristic of habituation. Evidence against simple neurotransmitter depletion as a causal factor in habituation also comes from work conducted in cultured *Aplysia* neurons (Eliot, Kandel, & Hawkins, 1994). The authors measured spontaneous miniature excitatory postsynaptic responses (mEPSCs) in motor neurons, which are the miniature depolarizations produced by spontaneous release of neurotransmitter and are reflective of presynaptic transmitter stores. It was shown that repeated presynaptic stimulation of the sensory neuron lead to decreases in postsynaptic EPSPs, but no changes in either the frequency or amplitude of mEPSCs. If habituation did lead to transmitter depletion then the frequency and/or the amplitude of spontaneous transmitter release, as assessed by the measurement of mEPSCs, should have been affected (Eliot et al., 1994). Since they were not, Eliot and colleagues (1994) concluded that transmitter depletion is not the primary mechanism of homosynaptic depression (the cellular analog of habituation). In *Drosophila melanogaster* three single-gene mutations (*dunce*, *turnip*, and *rutabaga*) have been found that affect both habituation and sensitization, as well as more complex associative learning (Duerr & Quinn, 1982). This suggests that the same genes and their products are somehow part of a mechanism involved in both simple and more complex learning. More recently two of these mutations, which affect the second messenger cAMP (cyclic adenosine monophosphate) have been shown to affect habituation of an escape response in *Drosophila* (Engel & Wu, 1996). Mutations of *rutabaga* which diminish cAMP synthesis result in reduced habituation rates, while mutations in *dunce* which increase cAMP levels result in increased habituation rates. In these experiments, spontaneous recovery and dishabituation were not significantly affected by the *dunce* and *rutabaga* mutations. Thus, in *Drosophila*, cAMP appears to play an important role in mechanisms underlying habituation while not playing a significant role in the mechanisms underlying dishabituation and spontaneous recovery (Engel & Wu, 1996).

Studying the mechanisms underlying habituation is useful for two reasons. First, understanding the basis of a widespread behavioural phenomenon like habituation is important in and of itself. Second, habituation can be used as a model to study different forms of memory. Generally, in simple system investigations, memory has been parsed in the time domain. At least two temporally constrained forms of memory have been defined: one form lasting a few minutes (short-term) and another lasting days or longer (long-term). This distinction is applicable to memory for both simple forms of learning such as habituation and sensitization, and more complex types of learning such as classical conditioning. Understanding the contribution of specific genes and their products to each of these processes has been the focus of our investigations.

The nematode *Caenorhabditis elegans* (*C. elegans*) has provided a valuable invertebrate model for the study of learning and memory, particularly for the study of habituation. Depending on training parameters, short-lasting and long-lasting forms of habituation can be studied.

C. elegans is a small (about one millimeter), hermaphroditic, soil-dwelling nematode (see Figure 1). There are a number of advantages that make *C. elegans* an excellent candidate for the study of learning and memory. The nervous system of *C. elegans* is very simple, consisting of 302 neurons that make approximately 10,000 chemical and electrical connections for which a complete wiring diagram is available (White, Southgate, Thomson, & Brenner, 1986). Furthermore, the *C. elegans* genome has been fully sequenced and the expression pattern of many genes in the organism are understood (Hodgkin, Horvitz, Jasny, & Kimble, 1998). In addition, the worm community makes literally thousands of genetic mutant strains available for analysis. The behavioural repertoire of *C. elegans* is complex enough to offer a number of interesting behaviors for study. In the laboratory worms swim

Figure 1. Schematic drawing of the nematode, *Caenorhabditis elegans*. The adult worm is approximately 1mm long and 40 μm wide.

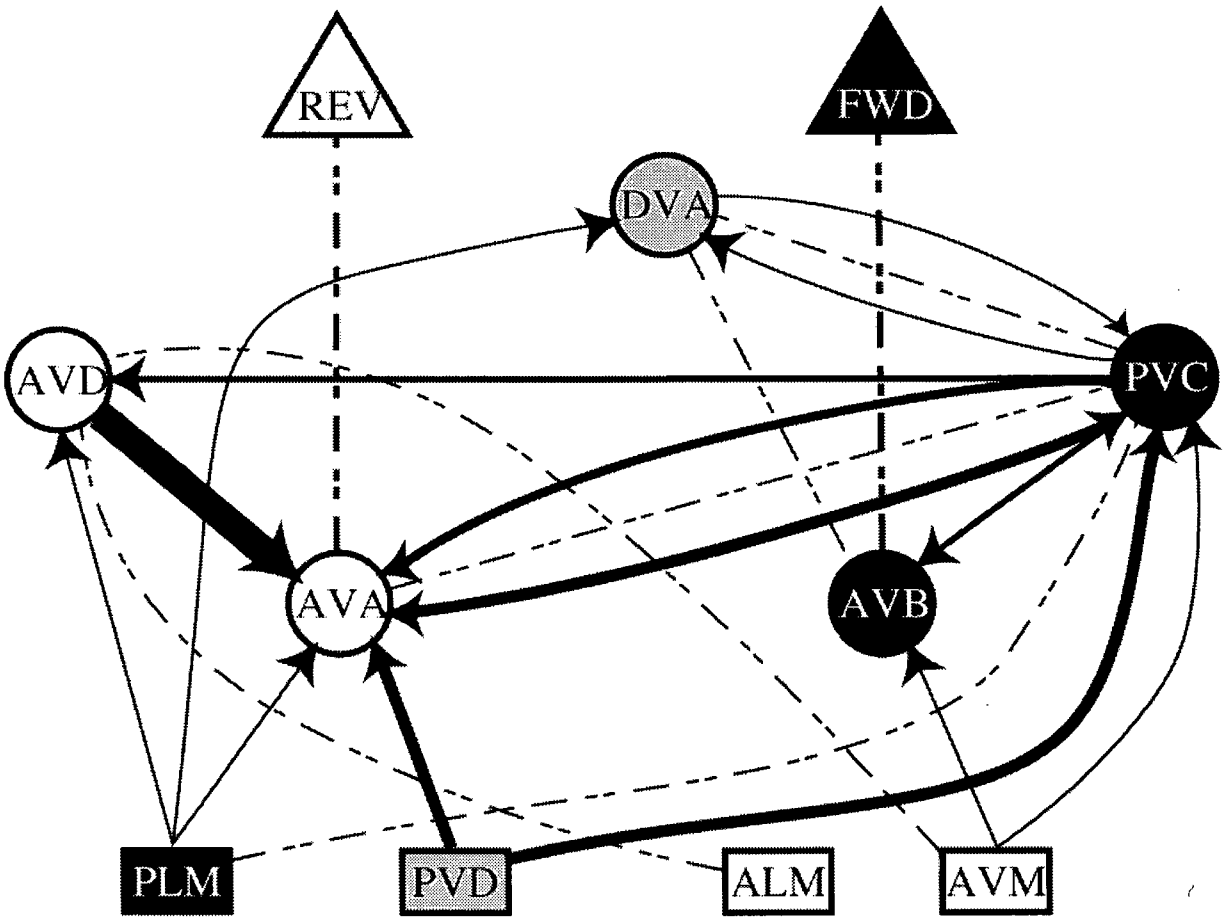

pharynx
intestine
head
tail
eggs
vulva
anus

forward along the surface of agar-filled petri dishes, using rhythmic co-ordinated contractions of dorsal and ventral muscle groups, resulting in smooth sinusoidal waves of forward locomotion. Worms will respond to a variety of stimuli by changing direction and by swimming backward (a reversal). Stimuli that produce reversals include touch, heat probes, some chemical compounds, and vibrations caused by the force of a mechanical tapper applied to the side of the dish. The response to a mechanical tap has been termed the tap withdrawal response (TWR) by Rankin, Beck, and Chiba (1990) and has proven to be important for studies of both short- and long-term memory.

The neural circuitry underlying forward and backward movement in response to tail touch and head touch respectively, was characterized by Chalfie and colleagues (1985). Starting with these two touch circuits, Wicks and Rankin (1995) identified the neural circuit of the TWR by laser ablating cells from the touch circuits and cells that synapse onto them. The tap-withdrawal circuit consists of five sensory neurons (2 PLM's in the tail, two ALM's in the anterior body, a single AVM located at midline), eight command interneurons (AVDs, AVAs, AVBs, and PVCs), and two pools of motor neurons (Figure 2). These neurons are also part of the touch circuits. In addition, the TWR circuit includes two PVD sensory neurons and a DVA interneuron. The force applied by the mechanical tapper activates both the head and tail touch circuits simultaneously so that the competing responses from these two circuits is integrated to produce a single behavior. The neurons of the TWR circuit make both electrical and chemical connections. The response itself is thought to be electrically driven, while modulation of the response is thought to occur at the chemical synapses (Wicks and Rankin, 1997).

Since the neural circuitry underlying the behavioural response used in all our experiments is well understood, experience-dependant plasticity of this behavior can only

Figure 2. The neural circuit for the tap-withdrawal response. The circuit consists of seven sensory neurons (rectangles), nine interneurons (circles), and two pools of motorneurons (triangles) that drive forward (FWD) or backward (REV) movement. All cells are bilateral except AVM and DVA, which are both single cells. Chemical connections are indicated by arrows, with the number of synaptic contacts proportional to the width of the arrows. Gap junctions are indicated by dotted lines. Cells shaded in black represent the tail-touch acceleration response circuit and cells in white represent the head-touch reversal circuit. Cells in grey represent cells that are involved in both circuits.


REV
FWD
DVA
AVD
PVC
AVA
AVB
PLM
PVD
ALM
AVM

occur in a handful of neurons. This is a major advantage, greatly simplifying the task of quantifying these changes. Knowledge of the neural circuitry combined with knowledge of the organisms' genome puts the *C. elegans* researcher in the unique position to study the role of specific gene products known to be expressed on specific neurons of an identified circuit involved in complex processes such as learning and memory.

C. elegans is capable of memory for learning associated with the tap withdrawal response. With repeated presentation of taps, habituation is evident as decreases in reversal magnitude (Rankin, Beck, & Chiba, 1990). Consistent with the rules of habituation outlined by Groves and Thompson (1970), the rate of habituation of the TWR in *C. elegans* is sensitive to the frequency of stimulation. In wild-type worms habituation occurs more rapidly at short inter-stimulus intervals (ISIs) (i.e., intervals of two or ten seconds) compared to long ISIs (i.e., intervals of sixty seconds). Spontaneous recovery from habituation is also affected by ISI, with animals recovering more rapidly from habituation with short ISIs than with long ISIs (Rankin and Broster, 1992). Thus, in *C. elegans* short-term memory for habituation training lasts less than fifteen minutes when trained with short ISIs and can last for one hour or longer when trained with long ISIs. The response decrements observed with repeated tap presentation in *C. elegans* are not due to fatigue for two reasons: First, presentation of a novel stimulus to a habituated animal immediately causes a return of the behavior to prehabituation levels, i.e., dishabituation (Rankin, Beck, and Chiba, 1990). Second, if the observed response decrements were in fact due to fatigue then the less decremented and therefore less fatigued group (i.e., those habituated at a 60s ISI) should recover more rapidly than the more decremented and therefore more fatigued group (i.e., those habituated at a 10s ISI). In fact the opposite is true, the less habituated group (60s ISI) recovers more slowly than the more habituated group (10s ISI). The effect of ISI on spontaneous recovery also argues against the

hypothesis that the sole mechanism of habituation is neurotransmitter depletion. The reasoning is very similar. Again, the less habituated group (i.e., those habituated at a long ISI) should recover faster. Presumably, there is less extensive depletion of neurotransmitter in this group. In fact, this group recovers more slowly.

ISI plays a crucial role in the kinetics of short-term habituation and the rate of spontaneous recovery. Rankin and Broster (1992) showed that it is ISI and not the number of stimuli or the amount of habituation that determines the rate of spontaneous recovery. In their experiments they found that once a worm had received sufficient stimuli so that its response had decremented to asymptotic levels for that ISI, the rate of spontaneous recovery from habituation was determined by the ISI of training. This suggested to them that the neurons underlying the behavior were in some way encoding the ISI of training in a way that set their rate of recovery. This led Rankin and Broster (1992) to the hypothesis that there are a number of different cellular processes underlying habituation, and that some are common to all stimulus protocols, while others are preferentially recruited by either long or short ISIs. Short-term memory for habituation is thought not to be a single process, but rather a family of ISI-dependent mechanisms that somehow encode the inter-stimulus interval of training. Thus, all short-term habituation cannot be explained by the same cellular mechanisms: some of the cellular processes may be triggered only by short ISIs (i.e., 10s), others may only be triggered by long ISIs (i.e., 60s), while some processes may be common to all ISIs.

In addition to short-term memory, with appropriate training, *C. elegans* also shows long-term memory (>24 hrs) for habituation (LTH) to tap. The paradigm now used to study long-term memory (Rose, Kaun, & Rankin, 2002) is based on a protocol originally developed by Beck and Rankin (1995, 1997). In *C. elegans* only distributed training leads to the formation of LTH (80 tap stimuli at a 60s ISI divided into four blocks of twenty stimuli

separated by 1-hour intervals). If the same number of stimuli (i.e., 80 taps) at the same ISI (60s) are given in one single block (massed training) then no significant LTH is obtained twenty-four hours later (Beck and Rankin, 1997; Rose et al., 2002). This is consistent with findings in other organisms, including *Drosophila* (Quinn & Dudai, 1976), *Aplysia* (Carew, Pinsker, & Kandel, 1972; Mauelshaugen, Sherff, & Carew, 1998) and humans (Ebbinghaus, 1885/1964). Long-term memory for distributed training only occurs when the stimuli within training blocks are given at a long ISI (60s) and not when given at a short ISI (10s) (Beck and Rankin, 1997; Rose et al., 2002), suggesting that the cellular mechanisms underlying habituation at short ISIs do not support a transition to long-term memory whereas those underlying habituation at long ISIs do. Long-term habituation in *C. elegans* has been shown to be protein synthesis dependant (Beck & Rankin, 1995; Rose et al., 2002). Animals generally respond to heat shock by making heat shock proteins and ceasing all other non-essential functions including the synthesis of other proteins (Schlesinger, Kelley, Aliperti, & Malfer, 1982). If heat shock is given during the 1-hour rest intervals between training blocks, then worms show no significant LTH. If on the other hand, heat shock is given prior to training or prior to retention testing, then there are no significant effects on LTH.

There are a number of experimental approaches that can be taken to study the mechanisms underlying short-term and long-term memory systems. A pharmacological approach involves drugs that may selectively affect stages of memory. Different drugs exert their effects through different neurotransmitter systems and act at different locations in the nervous system. Knowledge of these variables and their effects on learning and memory processes may help advance understanding of the neural and neurochemical processes underlying learning and different stages of memory. Another option is to take a developmental approach, determining at which stages of the nervous systems' development

various memory processes emerge. For example, Rankin and Carew (1987) showed that habituation of the siphon withdrawal reflex to different ISIs in *Aplysia* appeared at different times during the animal's juvenile life. The youngest animals only showed habituation to the shortest ISIs tested (1s) but not to longer ISIs (5s and 10s). As animals became progressively older they showed habituation to progressively longer ISIs. This suggested that the cellular processes underlying habituation to different ISIs appeared at different times during the nervous system's development and thus were dissociable. A third option is to take a genetic approach. Here, the object is to find gene mutations that selectively affect one or more stages of memory. Assuming knowledge of processes that these genes are involved in, this approach can provide clues to understanding the underlying mechanisms of learning and memory. The most well-known example of such a genetic analysis of memory can be seen in the fruit-fly, *Drosophila melanogaster*. In this species memory for a classically conditioned odour-avoidance response has been genetically dissected (Tully & Quinn, 1985). A number of mutations have been discovered that selectively affect different stages of memory, some affecting learning, others short-term memory, and yet others long-term memory. In fact the genetic approach taken in *Drosophila* has elucidated at least two additional unique memory phases, a middle-term memory phase, and an anesthesia-resistant form of long-term memory. So far there is supporting evidence for a unique middle-term memory in *Aplysia* (Sutton, Masters, Bagnall, & Carew, 2001) and *C. elegans* (see Steidl & Rankin, in press for a review). Thus an overview of the evidence from different species reveals that the traditional distinction between only two temporally constrained forms of memory, one short-term and one long-term, may in fact need to be modified.

The observation that the rate of spontaneous recovery is different following habituation at long and short ISIs was used to infer that different ISIs produce memory in different ways.

If there are in fact multiple types of short-term memory then we should find genetic mutations that selectively affect habituation at short ISIs, long ISIs, or more than one ISI. This has been a major focus of our lab. The job of choosing appropriate candidate genes involved in the mechanisms underlying short-term and long-term habituation is made easier by the fact that the neural circuit underlying the response to tap is known. The locus of the synaptic plasticity underlying habituation of the TWR has been hypothesized to be at the chemical synapses between the sensory neurons and the command interneurons (Wicks & Rankin, 1997). There is evidence to suggest that glutamate is the neurotransmitter used at these synapses (Lee, Sawin, Chalfie, Horvitz, & Avery, 1999; Hart, Sims, & Kaplan, 1995; Maricq, Peckol, Driscoll, & Bargman 1995; Brockie, Madsen, & Maricq, 1997) and a number of genes that encode for glutamate receptors expressed on both the sensory neuron and the command interneurons have been isolated. These include NMDA and non-NMDA-type glutamate receptors expressed on the interneurons as well as inhibitory glutamate-gated chloride channels expressed on both the interneurons and the sensory neurons. The effects of mutations in glutamate receptor genes in short and long-term memory have been studied. So far no obvious effect of NMDA receptor mutations on either short-term or long-term memory for habituation has been seen (Rankin, personal communication). Mutation in the non-NMDA receptor gene *glr-1* has no obvious effect on short-term memory but blocks long-term memory for habituation (Rose, Chen, Kaun, & Rankin, 2001).

The gene *avr-14* encodes an α-type subunit of a glutamate-gated chloride channel (GluCl). Glutamate-gated chloride channels are found only in invertebrates, including nematodes, insects, crustaceans, and molluscs, and so far at least have not been identified in vertebrates (Cully, Wilkinson, Vassilatis, Etter, & Arena, 1996). Common structural properties warrants classification of glutamate-gated chloride channels into the same

family containing GABA, glycine, and nicotinic acetylcholine receptors (Cully et al., 1994). Upon binding of glutamate to the receptor, ligand-gated chloride channels allow the passage of chloride ions (Dent et al., 2000). Using green fluorescent protein (GFP), Dent et al. (2000) showed that *avr-14* is expressed exclusively in a subset of about 40 extrapharyngeal neurons. Among these 40 neurons *avr-14* is expressed on the glutamatergic sensory neurons of the TWR circuit including, ALM, PLM, and PVD, where it may act as an autoreceptor. Because of its location on the sensory neurons of the TWR circuit, the gene product of *avr-14* could play a role in the plasticity, both short-term and long-term, of habituation of the TWR.

The focus of this thesis is *avr-14*, a gene encoding for an inhibitory glutamate-gated chloride channel expressed on the sensory neurons of the tap-withdrawal circuit. The goal of this thesis is to conduct a full analysis of both short-term and long-term memory for habituation in *avr-14* mutants. Analyses of the differences between *avr-14* and wild-type worms are expected to provide some insight into the mechanisms underlying both short-term habituation and long-term memory for habituation training.

General Methods

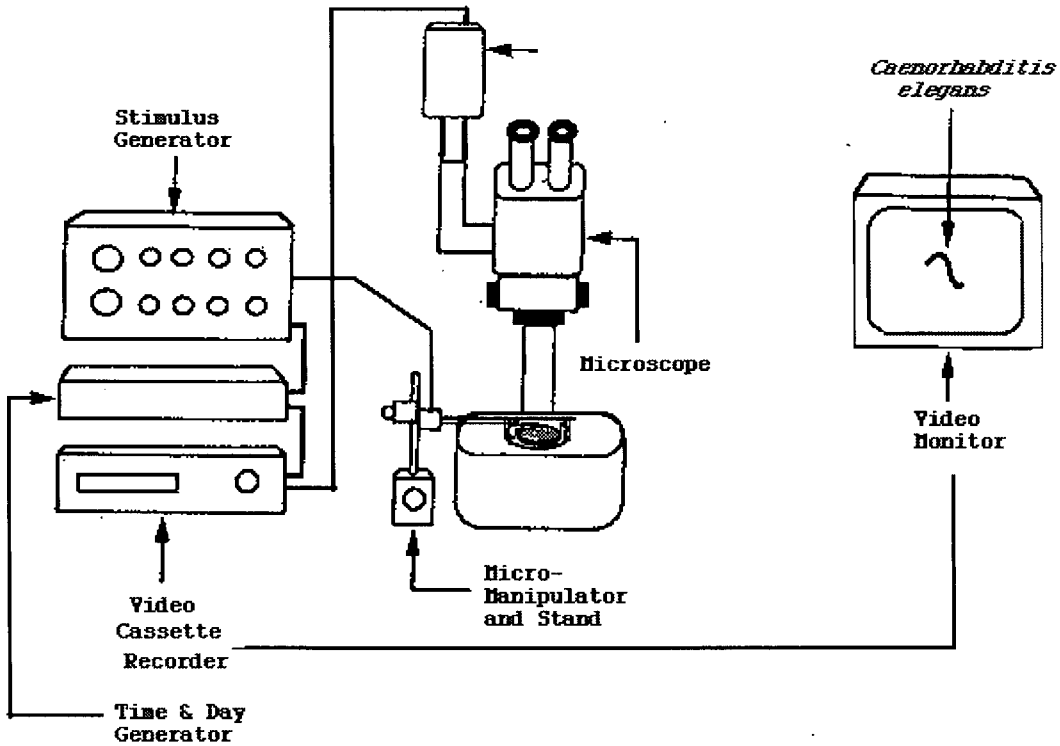
Subjects A grand total of 683 worms were used in Experiments 1-5. Varying numbers of wild-type and *avr-14* (da1371) worms were used in the five experiments. Wild-type Bristol N2 worms were obtained from the *Caenorhabditis elegans* Genetics Centre and *avr-14* (da1371) worms were generously provided by Dr. Joe Dent at McGill University. The strain used in all the experiments reported in this paper contains the *ad1302* allele of the *avr-14* gene which results from a missense mutation. This allele is thought to be functionally null for receptor function (Dent et al., 2000). All worms were synchronously grown at 20 °C on 5-cm Petri plates filled with 10-ml Nematode Growth Medium (NGM) agar and streaked with *E.*

coli strain OP50 as described by Brenner (1974). All testing was done in 4-day old adult worms.

Apparatus Animals were observed on the NGM agar with a stereomicroscope (Wild Leitz, Canada, Ltd., Model M3Z). All testing was recorded by a digital camera (Panasonic Digital 5100) connected to a VCR (Panasonic AG1960) and a monitor (NEC). In all recorded conditions a time-date generator (Panasonic WJ-810) superimposed the time and date on the video record as well as a digital clock to assist in precise stimulus delivery. Worms were individually tested on petri plates placed in a holder constructed from the lid of a petri plate that rested on the microscope stage. The holder was mounted onto a plastic rod, which in turn attached to a Marzhauser micromanipulator (Model MM33) which allowed for smooth, consistent movement of the plate in order to keep it in the video camera's field of view throughout testing. For delivery of the tap stimulus, a mechanical tapper, also attached to the plastic rod, consisting of a wire and an electromagnetic relay, was connected to a Grass S88 stimulator (Grass Instruments, Quincy, MA) to regulate stimulus delivery. The wire arm was positioned in such a way that tap stimuli were delivered to the side of the petri dish containing the worms, about halfway up the side of the dish. The stimulator was set to deliver taps of a 1-2 N force (See Figure 3 for a diagram of the apparatus).

Scoring Video records of testing sessions were scored using stop-frame video analysis. The magnitudes of individual reversals were traced onto acetate sheets for each worm. These tracings were then digitally scanned onto a Macintosh computer (DeskScan II) and measured with the public-domain NIH image program. Previous research (Wicks and Rankin, 1996) has shown that reversals and accelerations are mediated by different subsets of neurons and have different habituation kinetics. In these studies the focus was on reversals, so accelerations

Figure 3. The apparatus used to study habituation in *C. elegans*.


Caenorhabditis elegans
Stimulus Generator
Microscope
Video Monitor
Video Cassette Recorder
Micro-Manipulator and Stand
Time & Day Generator

were scored as missing data points, whereas pauses and no responses were scored as zeros.

Experiment 1: Short-term Habituation in *avr-14*

To assess the role of *avr-14* in short-term memory, *avr-14* and wild-type worms were compared at a variety of ISIs chosen to represent short, long, and intermediate ISIs: one group at a 10s ISI, one group at a 30s ISI, one group at a 45s ISI, and one group at a 60s ISI.

Method

Subjects A total of 87 *avr-14* (da1371) and N2 worms were used in this experiment. 22 worms per group at a 10s ISI, 20 worms per group at a 30s ISI, 22 worms per group at the 45s ISI, and 23 worms per group at the 60s ISI.

Procedure Worms were individually placed onto plain agar filled petri plates with no food and placed into the holder. After a six minute pre-plate period, intended to give the worm time to adjust to the new environment and recover from the transfer process, worms were subjected to a series of thirty tap stimuli at the appropriate ISI for the respective group.

Following the last tap of the series, three additional taps were presented to test for recovery from habituation. Regardless of ISI, these were always given at 30s, 5min, and 10min after the final tap stimulus of the series of thirty. For each ISI condition, *avr-14* (da1371) and wild-type control worms were always alternated in pairs.

Statistical Analysis The raw data across the series of thirty stimuli and the three recovery stimuli for each ISI for each strain were first standardized. Taking the first response in the series of habituation stimuli as the baseline, all subsequent responses were then re-expressed as a percentage of this baseline. The standardized data was then averaged into blocks of three responses each, and statistical analysis was performed on these data. Data for each ISI was analyzed separately. This experiment was clearly a two-way between-within repeated measures design, suggesting the use of an appropriate ANOVA model. Such a model does

however make certain assumptions about the data being analyzed. Chiefly among these is the assumption of homogeneity of covariance matrices. Preliminary testing using the Bartlett-Box test showed that this condition was not met in three of the four ISIs used in this experiment. Violation of this assumption means that neither the test on the between subjects factor nor the test on the interaction are valid. These were exactly the two hypothesis whose tests were most critical to the experimenter. Thus an alternate approach was taken. Profile Analysis is an extension of Hotelling's T^2 procedure that allows for testing the same hypotheses as the two-way between-within repeated measures ANOVA, but does not make the same assumptions (Morrison, 1990). This method is not affected by violation of the homogeneity of covariance matrices assumption and thus is both a more valid and more powerful test in this situation. The assumption of commensurability made by Profile Analysis was clearly met as all responses are expressed in the same units. Each of the three hypotheses tested by Profile Analysis are analogues of their repeated measures ANOVA counterparts. First, the hypothesis of Parallelism tests whether the profiles of means across the levels of the within-subjects factor in the two groups are similar or not. This is equivalent to the interaction between the between subjects factor and the within subjects factor in the ANOVA model. Second, the Levels hypothesis tests whether the group means are at the same level. This is akin to the test on the between subjects factor in ANOVA. Finally, the hypothesis of Flatness tests whether overall the slope of the profile across the repeated measures factor is significantly different from zero. This is equivalent to the test on the within subjects factor in the ANOVA model. As in ANOVA, rejection of the null hypothesis on any of these is followed by appropriate follow-up analyses and rejection of the null hypothesis of Parallelism precludes analysis of both the Levels and the Flatness hypothesis (as does a significant interaction in ANOVA). In the case of significance on any of the three hypotheses, a series of confidence intervals was

constructed on each of the contrasts being tested by the overall test. For each of the four ISIs, the data was divided into three components: the initial response decrement (the exponential drop in responding; blocks 1-3 which included the first 9 taps of the series), asymptotic response levels (blocks 4-10 which included taps10 to 30), and spontaneous recovery (recovery taps presented at 30s, 5 min, and 10 min after the thirtieth tap of the series). Both the initial response decrement and the asymptotic response levels were analyzed using Profile Analysis. The three hypotheses tested by this method were employed as seen fit for the situation (see below for details).

For consistency between the various ISI groups, only the 5 min and 10 min recovery points were included in the analysis. This was because the 30s recovery point was equal to another stimulus in the 30s ISI group, less than another stimulus in the 45s and 60s ISI groups, and more than another stimulus in the 10s ISI group. It was recognized that comparing mean recovery levels between strains at each ISI was not appropriate, since particularly at the short ISIs worms were not entering this phase of the experiment with the same level of habituation. For example, at the 10s ISI asymptotic levels in *avr-14* worms were lower than in N2 worms (see below), so that mean recovery levels may not have been the most accurate method of assessing the rate of spontaneous recovery. In order to accommodate the differences in asymptotic response levels, habituated response levels (Xhab; the mean of the last three responses in the series of thirty taps; Rankin & Broster, 1992) for each worm were subtracted from individual standardized responses to recovery stimuli. Two specific comparisons at each ISI were of interest: mean recovery at 5 min in *avr-14* and N2 worms, and mean recovery at 10 min in *avr-14* and N2 worms. Since these were the only comparisons of interest, the method of planned orthogonal contrasts (POC's) was employed.

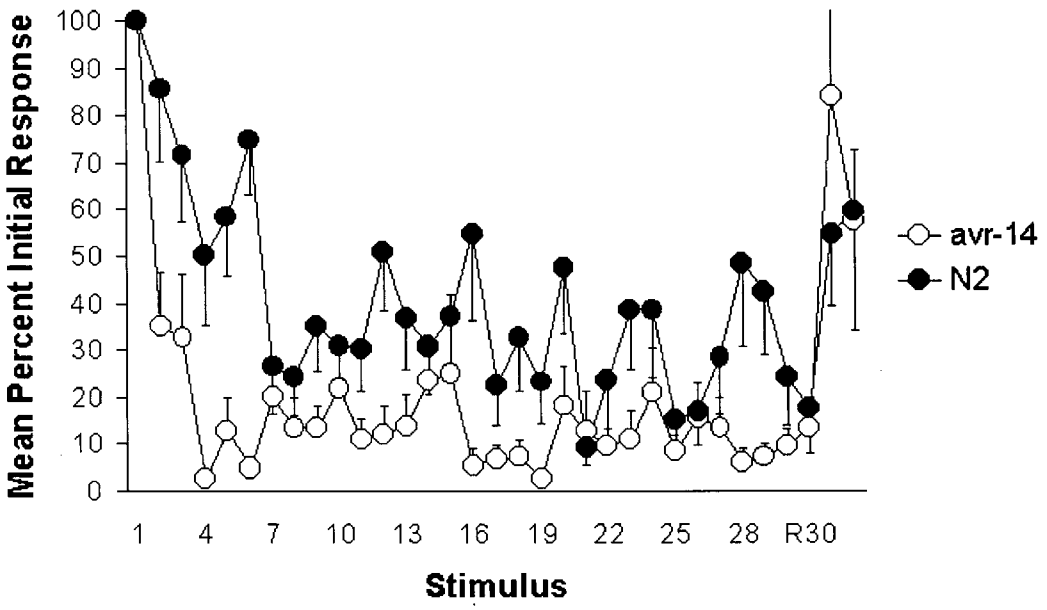
Results

Habituation

10s ISI. Response magnitudes expressed as a percentage of the first response are shown in Figure 4. Analysis of the first component (initial response decrements) revealed significant deviation from Parallelism (F (2, 41) = 28.12, $p < 0.001$), indicating that the profile of the two groups in this phase of the experiment was not equal. The rate of response decrement in *avr-14* was significantly different than in N2. Confidence intervals on the two contrasts (block1 vs. block 2 and block 2 vs. block3) showed that the difference in mean response magnitude between the two strains was significantly larger at block 2 compared to block1 ($p < 0.05$), and also significantly smaller at block 3 compared to block 2 ($p < 0.05$). Thus, the rate of response decrement across the first three blocks of stimuli was significantly more rapid in *avr-14* worms compared to wild-type worms.

Analysis of asymptotic response levels (blocks 4-10) revealed, as expected from inspection of Figure 4 (bottom), no significant deviations from Parallelism (F (6, 37) = 0.0624, $p > 0.1$). A test of the Levels hypothesis revealed significant overall differences in mean response magnitudes across blocks 4 to 7 between the two strains (t (42) = 4.32, $p < 0.001$). Thus overall asymptotic response levels were significantly lower in *avr-14* worms than in N2 worms. Finally, a test of the Flatness hypothesis revealed no significant overall deviation from Flatness across the two strains (F (6, 37) = 0.7067, $p > 0.1$). This indicates that there were no significant changes in mean response magnitude across this phase of the experiment. Thus both strains had in fact reached asymptotic response levels characterized by no further response decrements, but asymptotic levels were lower in *avr-14*. One striking feature of the data shown in Figure 4 (bottom) is the difference in the size of the error bars (standard error of the mean) between the two strains in the initial response decrement phase

Figure 4. Habituation at a 10s ISI in *avr-14* (n=22) and N2 (n=22) worms. Top: Mean standardized reversal magnitudes (± SEM) across thirty stimuli presented at a 10s ISI, and three recovery stimuli presented 30s (R30), 5 min, and 10 min after the final stimulus of the habituation series. All data were standardized with respect to the initial response. Bottom: Mean standardized reversal magnitudes (± SEM) across thirty stimuli at a 10s ISI grouped in blocks of three stimuli each (i.e., block1 represents the average response across stimuli 1-3, block 2 across stimuli 4-6, etc. Block 11 is the average response across recovery taps given at 5 min and 10 min after the final stimulus of the habituation series).


Mean Percent Initial Response
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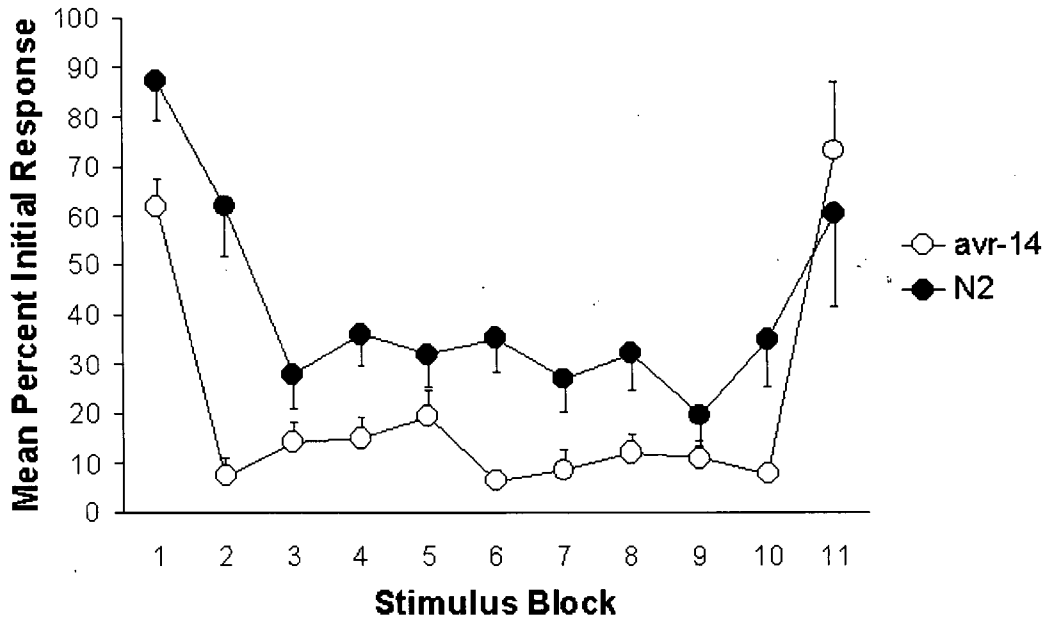
R30
Stimulus
avr-14
N2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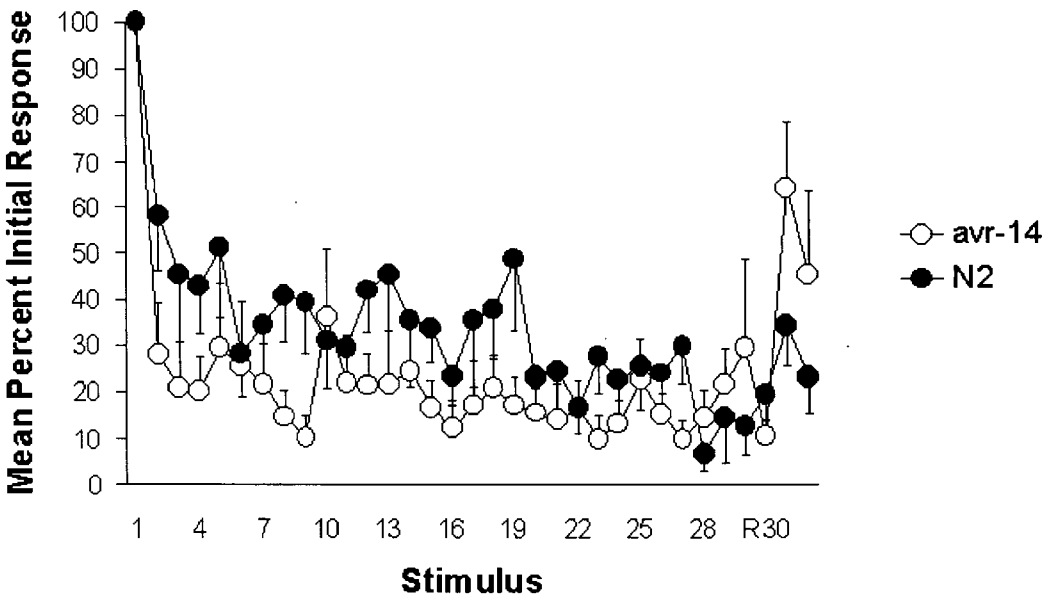
11
Stimulus Block
avr-14
N2

and the asymptotic response level phase. A F-test for two independent variances showed that the overall variance across blocks 1 to 10 was significantly lower in *avr-14* worms compared to wild-type (F (21, 21) = 2.621, $p < 0.05$).

30s ISI. Response magnitudes expressed as a percentage of the first response are shown in Figure 5. Analysis of the initial response decrement revealed no significant departure from Parallelism (F (2, 37) = 0.334, $p > 0.05$), indicating that the rate of habituation was not significantly different between *avr-14* and N2 worms. Further, there were no significant differences in overall response magnitude across the three blocks between the two strains, as revealed by a test of the Levels hypothesis (t (38) = 0.318, $p > 0.05$). Finally, as expected, and as is obvious in Figure 5 (bottom), there was significant departure from Flatness (F (2, 38) = 27.115, $p < 0.05$). This merely indicates that both strains were in fact habituating.

A different approach was taken for the analysis of asymptotic response levels at the 30s ISI than at the 10s ISI. Inspection of Figure 5 (bottom) shows that the profiles for the two strains cross between blocks 9 and 10. This would have most certainly resulted in rejection of the null hypothesis of Parallelism between the two profiles. This would have not been very revealing, so instead only the Flatness and the Levels hypothesis were tested. A test of the Levels hypothesis revealed significant differences in overall mean response magnitudes between the two strains (t (38) = 2.657, $p < 0.05$) indicating that overall mean response magnitudes across blocks 4-10 were significantly lower in *avr-14* worms compared to wild-type. Finally, a test of the Flatness hypothesis revealed no significant departures from Flatness across the two strains (F (6, 34) = 2.4056, $p > 0.05$). This means that both strains had in fact reached asymptotic response levels and did no show any further response decrements, but asymptotic response levels were lower in *avr-14* worms.

Figure 5. Habituation at a 30s ISI in *avr-14* (n=20) and N2 (n=20) worms. Top: Mean standardized reversal magnitudes (± SEM) across thirty stimuli presented at a 30s ISI, and three recovery stimuli presented 30s (R30), 5 min, and 10 min after the final stimulus of the habituation series. All data were standardized with respect to the initial response. Bottom: Mean standardized reversal magnitudes (± SEM) across thirty stimuli at a 30s ISI grouped in blocks of three stimuli each (i.e., block1 represents the average response across stimuli 1-3, block 2 across stimuli 4-6, etc. Block 11 is the average response across recovery taps given at 5 min and 10 min after the final stimulus of the habituation series).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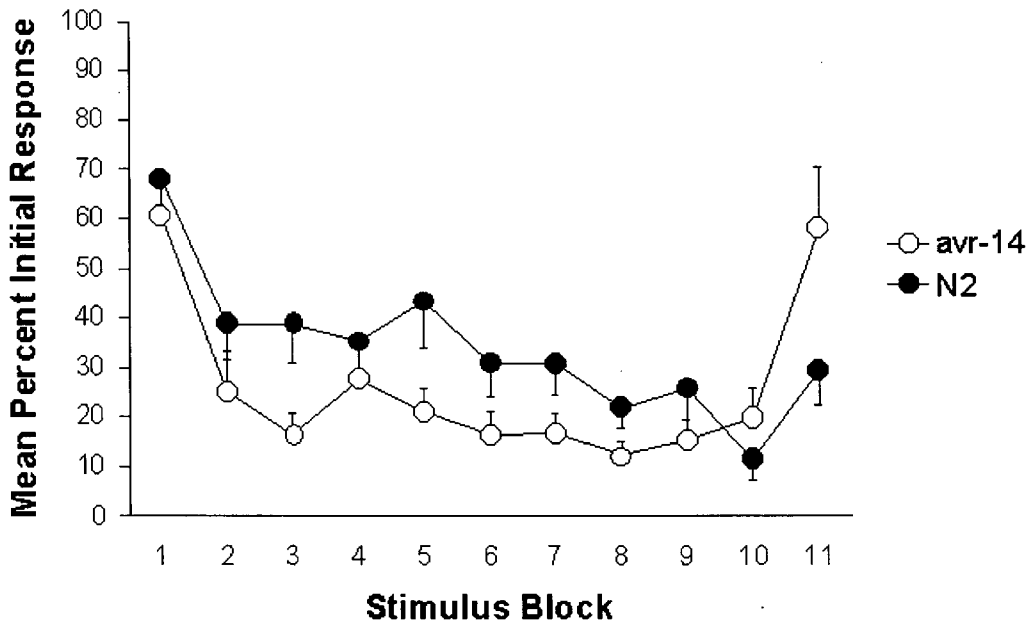
22
25
28
R30
Stimulus
avr-14
N2


Mean Percent Initial Response
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11
Stimulus Block
avr-14
N2

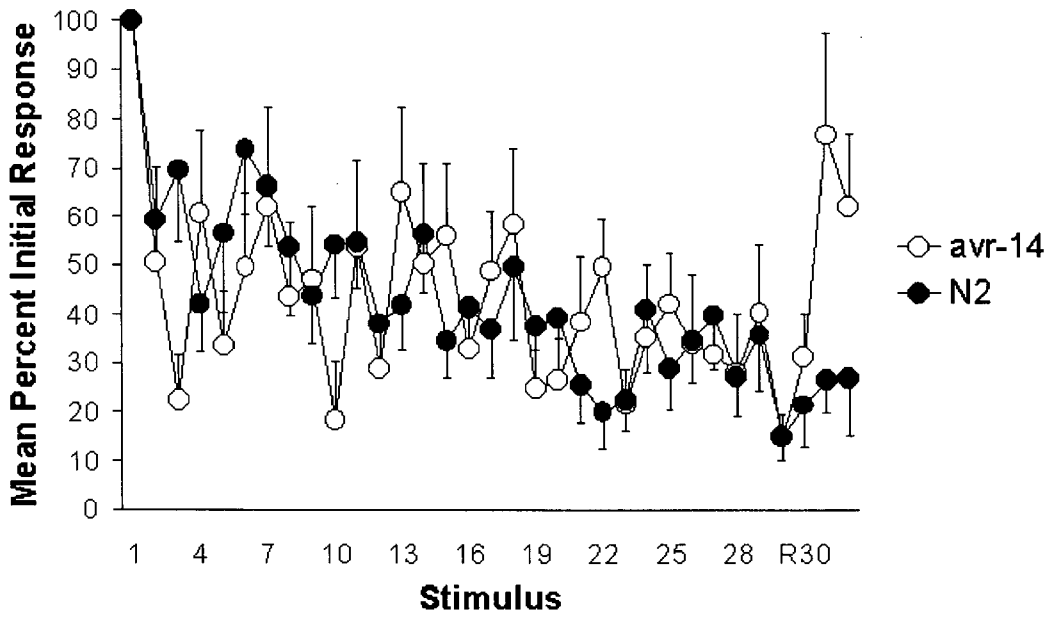
It appeared again, like at the 10s ISI, that error bars in *avr-14* worms were smaller compared to those in wild-type (see Figure 5, bottom). However, at the 30s ISI, unlike at the 10s ISI, the difference in overall variance was not statistically significant (F (19, 19) = 1.3027, $p > 0.05$).

45s ISI. Response magnitudes expressed as a percentage of the first response are shown in Figure 6. Inspection of Figure 6 (bottom) shows that the profiles of the two strains cross repeatedly across the 10 blocks of training. This condition would certainly have lead to departures from Parallelism if that hypothesis were tested. Thus, using the same reasoning as for the analysis of asymptotic response levels at the 30s ISI, the hypothesis of Parallelism was not tested for the initial response decrement phase or the asymptotic response levels at the 45s ISI.

Analysis of initial response decrements revealed no significant differences for the Levels hypothesis (t (42) = 0.6965, $p > 0.05$). Thus overall mean response levels between *avr-14* and wild-type worms were not significantly different in this phase of the experiment. There was however, not surprisingly, significant departure from Flatness (F (2, 41) = 3.421, $p < 0.05$). This of course only indicates that both strains were showing significant response decrements (habituation) over the first three blocks of training.

Analysis of asymptotic response levels (blocks 4-10) revealed no significant differences in overall response levels between the two strains, as indicated by a test of the Levels hypothesis (t (42) = 0.2798, $p > 0.05$). There was however significant departure from Flatness (F (6, 37) = 2.532, $p < 0.05$). This indicates that both strains were still showing response decrements during this phase of the experiment. Follow-up analysis to assess where this deviation from Flatness was coming from revealed that this departure was specifically due to larger mean response levels across both strains in block 5 compared to block 10 ($p < 0.05$).

Figure 6. Habituation at a 45s ISI in *avr-14* (n=22) and N2 (n=22) worms. Top: Mean standardized reversal magnitudes (± SEM) across thirty stimuli presented at a 45s ISI, and three recovery stimuli presented 30s (R30), 5 min, and 10 min after the final stimulus of the habituation series. All data were standardized with respect to the initial response. Bottom: Mean standardized reversal magnitudes (± SEM) across thirty stimuli at a 45s ISI grouped in blocks of three stimuli each (i.e., block1 represents the average response across stimuli 1-3, block 2 across stimuli 4-6, etc. Block 11 is the average response across recovery taps given at 5 min and 10 min after the final stimulus of the habituation series).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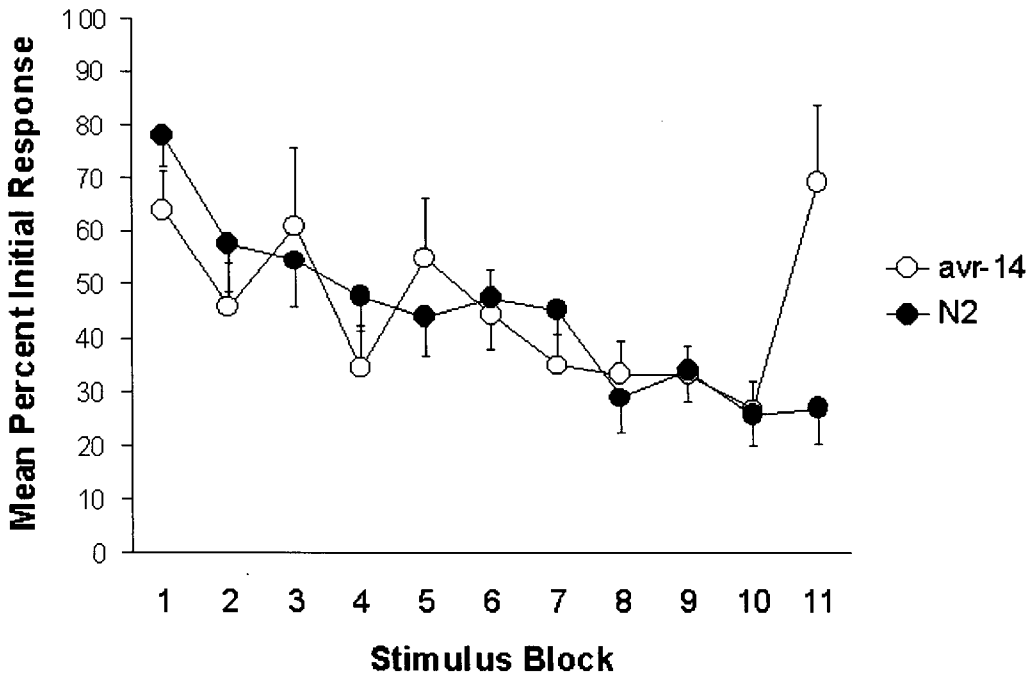
R30
Stimulus
avr-14
N2


Mean Percent Initial Response
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11
Stimulus Block
avr-14
N2

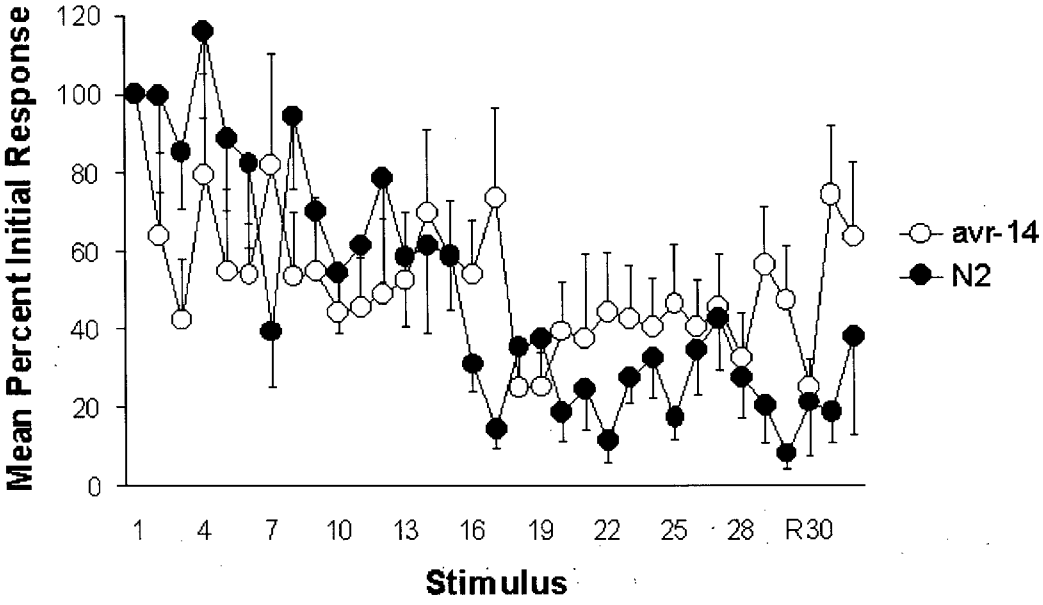
From this it appears that at a 45s ISI, neither strain had reached asymptotic response levels within the thirty stimuli presented.

Finally, inspection of Figure 6 (bottom) indicates that the size of the error bars between the two strains appear approximately equal across both the initial response decrement phase and asymptotic response levels phase. Statistical analysis did indeed support this assertion ($F(21, 21) = 1.196$, $p > 0.05$).

60s ISI. Response magnitudes expressed as a percentage of the first response are shown in Figure 7. Analysis of the initial decrement in responding revealed no significant departure from Parallelism ($F(2, 43) = 1.103$, $p > 0.05$), indicating that there were no significant differences in the rate of initial response decrement between the two strains. Furthermore, there were no significant differences in the overall mean level of responding between the two strains, as indicated by a test of the Levels hypothesis ($t(44) = 1.679$, $p > 0.05$). Also, there were no significant departures from Flatness ($F(2, 43) = 2.06$, $p > 0.05$). This means that at the 60s ISI neither *avr-14* nor N2 worms showed significant decrements in responding over the first three blocks of stimuli.

Inspection of asymptotic response levels in Figure 7 (bottom) shows that the profiles for the two strains cross between blocks 5 and 6, due to an unusually large response decrement in N2 worms at this point (see below for a detailed discussion). This condition would have certainly lead to significant deviations from Parallelism, which in turn would have precluded tests of the Levels and Flatness hypothesis, which seem more appropriate. A test of the Levels hypothesis revealed a significant difference in overall mean response magnitudes over this phase of the experiment ($t(44) = 2.308$, $p < 0.05$). At the 60s ISI, unlike any of the other ISIs, this difference was due to overall higher response levels in *avr-14* worms compared to wild-type. Further, a test of the Flatness hypothesis revealed significant departures from Flatness

Figure 7. Habituation at a 60s ISI in *avr-14* (n=23) and N2 (n=23) worms. Top: Mean standardized reversal magnitudes (± SEM) across thirty stimuli presented at a 60s ISI, and three recovery stimuli presented 30s (R30), 5 min, and 10 min after the final stimulus of the habituation series. All data were standardized with respect to the initial response. Bottom: Mean standardized reversal magnitudes (± SEM) across thirty stimuli at a 60s ISI grouped in blocks of three stimuli each (i.e., block1 represents the average response across stimuli 1-3, block 2 across stimuli 4-6, etc. Block 11 is the average response across recovery taps given at 5 min and 10 min after the final stimulus of the habituation series).


Mean Percent Initial Response
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Stimulus
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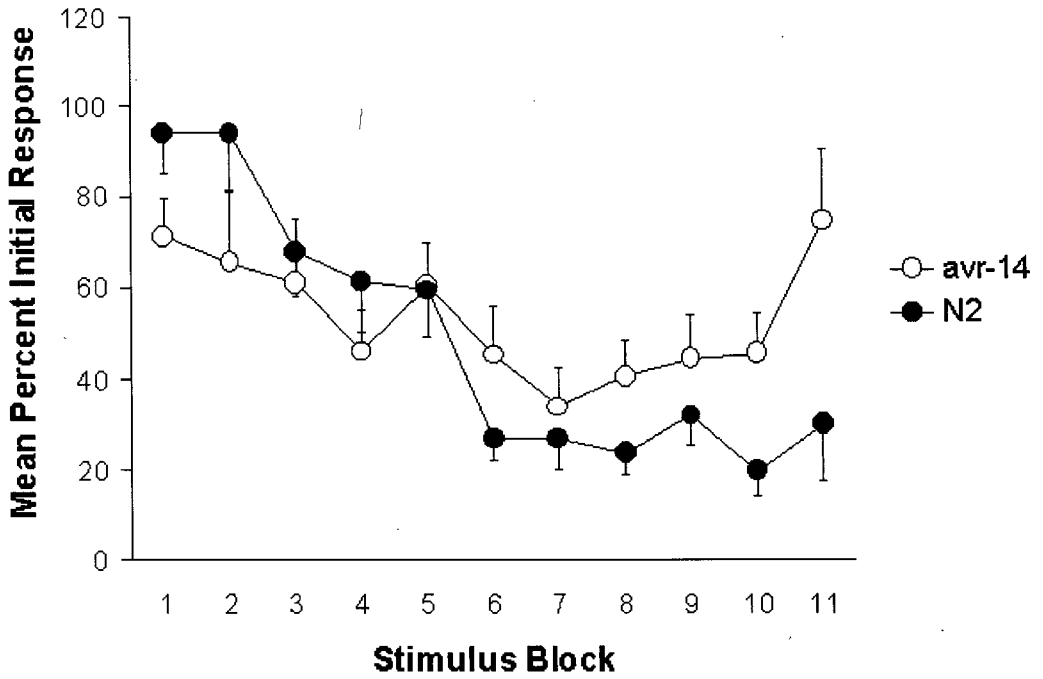
N2


Mean Percent Initial Response
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11
Stimulus Block
avr-14
N2

(F (6,39) = 3.398, $p < 0.05$). Follow-up analysis to assess where this deviation from Flatness was coming from revealed that this departure was specifically due to the larger mean response levels across both strains in block 5 compared to block 6 ($p < 0.05$).

This difference may also have been due to the large decrease in mean response levels occurring in wild-type worms at this time point. Thus, it is not clear how the departures from Flatness observed during asymptotic levels of responding at the 60s ISI should be interpreted; whether neither strain reached asymptotic response levels between blocks 4 to 10 of the experiment or whether this was merely due to an apparent anomaly that wild-type worms were showing. These possibilities will be discussed further below.

Finally, Figure 7 (bottom) also shows that there are no obvious differences in the size of the error bars between the two strains at the 60s ISI. Statistical analysis supported this conclusion, showing no significant difference in overall variability between the two strains (F (22, 22) = 1.05, $p > 0.05$).

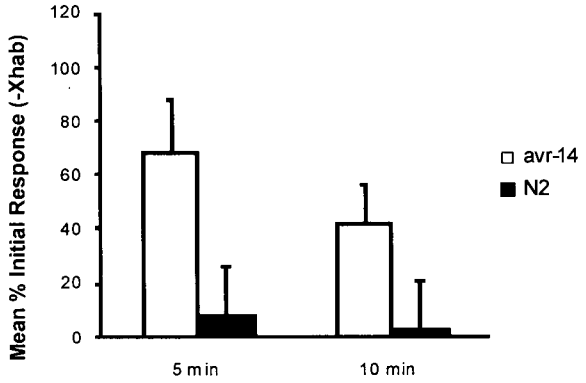
Spontaneous Recovery

10s ISI. Figure 8a shows spontaneous recovery expressed as a percentage of initial response magnitude at 5 and 10 minutes in both *avr-14* and N2 worms. Planned orthogonal contrast on mean recovery levels between the two strains revealed significantly higher recovery levels in *avr-14* at 5 minutes (F (1, 84) = 6.072, $p < 0.05$) but not at 10 minutes (F (1, 84) = 2.846, $p > 0.05$). Thus, it appears that spontaneous recovery occurred at a more rapid rate in *avr-14* compared to N2 when trained with 10s ISI.

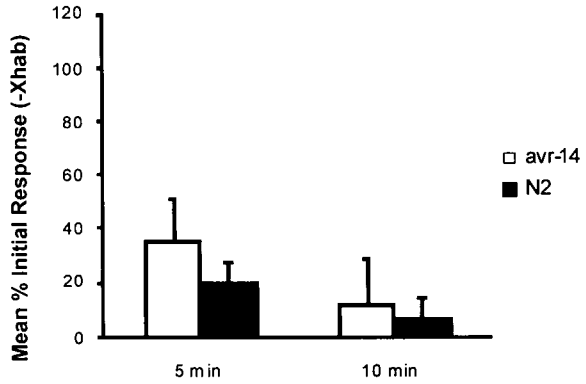
30s ISI. Figure 8b shows spontaneous recovery expressed as a percentage of initial response magnitude at 5 and 10 minutes in *avr-14* and N2 worms. Planned orthogonal contrasts on mean recovery levels between the two strains did not reveal significantly different recovery levels at 5 minutes (F (1, 76) = 0.763, $p > 0.05$) or at 10 minutes (F (1, 76) = 0.115,

Figure 8. Spontaneous Recovery from habituation plotted as a mean percentage of the initial response . (± SEM) for worms tested at 5 min and 10 min after habituation to 30 stimuli at various ISIs. For each worm the habituated response (Xhab, the mean of the last three habituation responses) was subtracted from each recovery point. A: recovery at a 10s ISI; B: recovery at a 30s ISI; C: recovery at a 45s ISI; D: recovery at a 60s ISI.

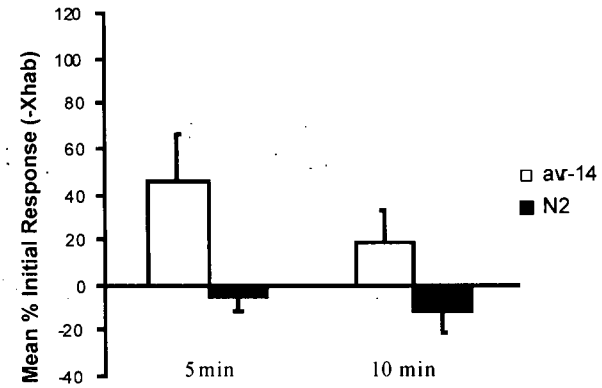
A: 10s ISI



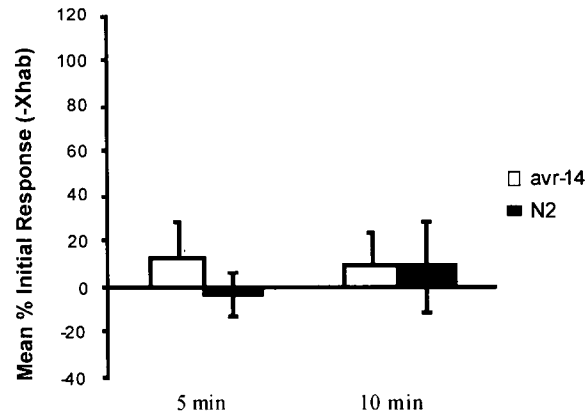
B: 30s ISI



C: 45s ISI



D: 60s ISI



$p > 0.05$). It appears that there were no differences in the rate of spontaneous recovery between *avr-14* and N2 worms when trained with a 45s ISI.

45s ISI. Figure 8c shows spontaneous recovery expressed as a percentage of initial response magnitude at 5 and 10 minutes in *avr-14* and N2 worms. Planned orthogonal contrasts on mean recovery levels between the two strains at 5 and 10 minutes revealed significantly higher recovery levels at 5 minutes (F (1, 84) = 7.192, $p < 0.05$) in *avr-14* worms compared to wild-type worms, but no differences at 10 minutes (F (1 ,84) = 2.489, $p > 0.05$). Inspection of Figure 8c reveals a trend of increasing mean recovery levels from 5 to 10 minutes in *avr-14* worms while there appears to be no difference in wild-type worms between 5 and 10 minutes recovery. In fact wild-type worms do not appear to show any spontaneous recovery at a 45s ISI. On the surface this may be taken as evidence that spontaneous recovery is more rapid in *avr-14* worms compared to wild-type worms at a 45s ISI. However the fact that wild-type worms showed no significant recovery at either 5 or 10 minutes was unexpected. Previous research indicates that there should be at least some recovery at 10 minutes when trained with a 45s ISI (Rankin & Broster, 1992). The fact that wild-type worms were behaving out of the ordinary may be distorting the differences seen in spontaneous recovery between the two strains when trained with a 45s ISI. The possibility that statistically significant differences at 10 minutes were due to wild-type worms not showing recovery, as opposed to *avr-14* showing enhanced recovery, cannot be ruled out.

60s ISI. Figure 8d shows spontaneous recovery expressed as a percentage of initial response magnitude at 5 and 10 minutes in both *avr-14* and N2 worms. Planned orthogonal contrasts on mean recovery between the two strains revealed no significant differences in mean recovery levels between *avr-14* and N2 worms at 5 minutes (F (1, 88) = 0.569, $p > 0.05$)

or at 10 minutes (F (1,88) = 0.0029, $p > 0.05$). Thus it appears that there were no differences in the rate of spontaneous recovery between *avr-14* and N2 worms when trained with a 60s ISI

Discussion

Habituation curves for both strains at all ISIs tested showed the classic pattern of habituation; high response levels at first, followed by a rapid exponential drop in response magnitude, followed by asymptotic response levels characterized by no further decrements in response magnitude. Across both strains, these factors varied with the ISI used during training. Initial response decrements were more rapid and asymptotic levels were lower when trained with a short ISI (10s and 30s) compared to a long ISI (45s and 60s). In fact, at the longest ISI (60s) neither strain appeared to show significant response decrements across the first three blocks of stimuli, and neither strain appeared to reach asymptotic response levels within the ten blocks of stimuli at either the 45s or 60s ISI. The apparent lack of asymptotic response levels at the 60s ISI came as a surprise and requires some clarification.

Mutations in the *avr-14* gene appeared to predominantly affect habituation at short ISI but not at long ISIs. Effects were most pronounced at the 10s ISI, the shortest ISI used in this experiment. At this ISI, initial response decrements were significantly more rapid in *avr-14* worms and asymptotic response levels were significantly lower. At the 30s ISI there were no significant differences in the initial rate of habituation but asymptotic response levels were significantly lower in *avr-14* worms compared to wild-type. Further, the overall level of variability in *avr-14* worms was significantly lower at the 10s ISI compared to wild-type. At the 30s ISI, though not significant, there was a clear trend of decreased variability as well. At the longer ISIs (45s and 60s) the two strains appeared more similar. At the 45s ISI, neither the initial rates of habituation nor the asymptotic response levels were significantly different between the two strains. At the 60s ISI there were no differences in the rate of habituation

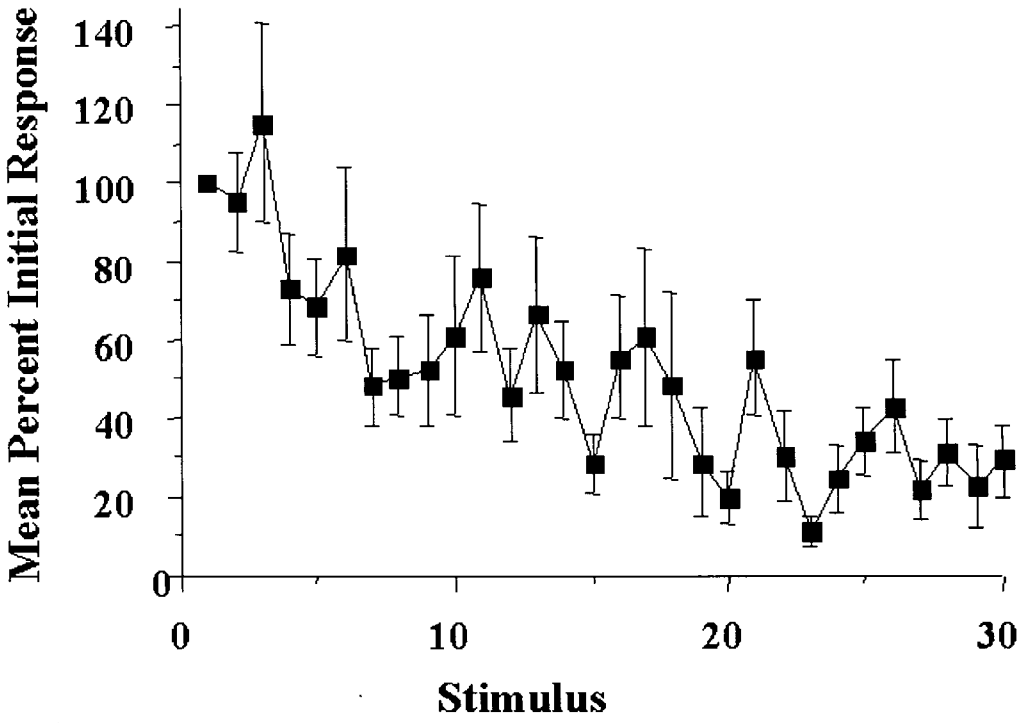
between the two strains. In fact there was no evidence of a large initial response decrement in either strain compared to the other ISIs. This was not an unusual result as habituation at a 60s ISI in *C. elegans* is characterized by gradual decrements in responding (Rankin & Broster, 1992). Perhaps the choice of defining the initial response decrement phase as the first three blocks of stimuli was not as appropriate for the 60s ISI as it was for all other ISIs. Adjusting the duration of this phase to for example the first four blocks of stimuli may have provided a different picture. For the sake of consistency across ISIs such an adjustment was not applied in the current experiment. Asymptotic levels of responding at the 60s ISI were the most difficult to interpret. Statistically there was a significant difference in the overall levels of responding between *avr-14* and wild-type worms. Mean response levels were higher on average in *avr-14* worms in this phase of the experiment. Generally, wild-type worms show a more gradual decline in responding when trained with a 60s ISI (Rankin & Broster, 1992). In this experiment a large decrease in mean response levels was observed between blocks five and six (see Figure 7, bottom). It is not understood why this occurred but it is clearly at odds with previous data obtained in wild-type worms. It is unlikely that this effect would be observed again if the experiment were replicated. Its presence does however cause some problems for interpretation of the data, as it is distorting the results. One of these problems is significantly higher asymptotic response levels in *avr-14* compared to wild-type worms. If as is usually observed, decrements in N2 occurred more gradually, this difference would not have been expected to be statistically significant. The second problem is a departure from Flatness in asymptotic response levels across both strains. Essentially, this implies that neither strain did in fact reach asymptotic response levels. Follow-up analysis clearly showed however that this departure from Flatness was specifically due to the response decrement in N2 worms between blocks 4 and 5. For comparison purposes, habituation data at a 60s ISI in N2 worms

from previous experiments are illustrated in Figure 9 (Rankin & Broster, 1992). It is clear from this figure that the current 60s ISI data obtained in wild-type worms differs somewhat from previous data. If the behavior observed in wild-type worms was in fact an anomaly, and the observed differences were in fact due to this unusual pattern of responding, then it can be concluded that there were no differences in either the initial rate of habituation nor asymptotic response levels between the two strains when trained with a 60s ISI.

The effects of the *avr-14* mutation on spontaneous recovery were unclear. At the shortest ISI tested (10s) the level of spontaneous recovery was higher in *avr-14* worms than in wild-type at five minutes but not at ten minutes. At the 30s ISI there were no differences. At the 45s ISI recovery levels were higher at five minutes but not at ten minutes. At the 60s ISI the rate of spontaneous recovery was again not different in *avr-14* worms compared to wild-type worms. The largest effects of the mutation on spontaneous recovery were clearly at the 10s ISI. However, at the 10s ISI wild-type worms showed very little recovery. Thus the differences between the two strains at the 10s ISI may have in fact been due to the fact that wild-type worms did not show any significant recovery. It also needs to be pointed out that previous investigations of spontaneous recovery in *C. elegans* have chosen longer time intervals at which to measure recovery from habituation (i.e., 10 min, 20 min, and 30 min; see Rankin & Broster, 1992). It may be that the effects of ISI on the rates of spontaneous recovery in wild-type worms are more obvious at these time points than the ones used in the current experiment. Perhaps potential differences in the rate of spontaneous recovery between the two current strains may also have been more obvious at these time points.

Overall, it appeared that the *avr-14* mutation affected both the rate and level of habituation as well as spontaneous recovery at a 10s ISI, only the final level of habituation at a 30s ISI, neither aspect of habituation, but somewhat the rate of spontaneous recovery at a 45s

Figure 9. Mean standardized reversal magnitudes (± SEM) across thirty stimuli presented at a 60s ISI in N2 worms (n=20).


Mean Percent Initial Response
140
120
100
80
60
40
20
0
0
10
20
30
Stimulus

ISI, and neither aspect of habituation nor spontaneous recovery at a 60s ISI.

The results obtained with the 10s ISI suggest a somewhat paradoxical effect on short-term memory for habituation. On the one hand, short-term memory during the habituation protocol appears to be superior, as indicated by more rapid habituation and lower asymptotic response levels. On the other hand, retention of habituation training, as indicated by the rate of spontaneous recovery, appeared to be compromised in *avr-14* worms. In other words, *avr-14* worms acquired habituation more rapidly when trained with a 10s ISI, but showed poorer retention of what was learned (the apparent insignificance of taps). It was as if the usual relationship between the rate of habituation and the rate of spontaneous recovery was actually strengthened. This apparent paradox is not without parallels in other areas of memory research on more complex types of learning. For example, in humans it has been shown that acquisition performance is in fact not a good indicator of learning (Schmidt & Bjork, 1992). Though the tasks used in these experiments were obviously more complex than simple response habituation, one result that was consistently observed was that factors which slowed acquisition of the task (both verbal and motor tasks were used) actually lead to superior retention of what was learned during the acquisition phase. Though these differences in retention were much larger when tested days after the acquisition phase, there was a trend of the same differences as little as ten minutes after the acquisition phase. Thus, this principle of faster acquisition leading to poorer retention appears to apply to both simple and complex types of learning. In *C. elegans* a mutation that results in even more rapid acquisition at short ISIs results in even poorer levels of retention than those usually observed at short ISIs.

The results obtained in this experiment are thought to provide support for the hypothesis of multiple ISI-dependant short-term memory systems. *avr-14*, and the mechanisms it is involved in, appear to be an example of a cellular process that is

preferentially recruited by training with short ISIs. Where exactly the border between short and long ISIs lies is not known, but based on the results obtained it appears to be somewhere around 30s. At the 45s ISI the effects of the mutation on habituation seem to have disappeared. At the 30s ISI effects are still evident, albeit to a lesser extent than at the 10s ISI, suggesting that the border may lie somewhere above 30s. It is likely that the cut-off point is closer to 30s than to 45s. Testing habituation at ISIs between 30s and 45s is expected to reveal where exactly the cutoff point between short and long ISIs lies.

The next step in pursuing the hypothesis of multiple short-term memory systems is understanding the cellular processes that *avr-14*, and for that matter any other mutation with similar ISI-dependant effects, are involved in. This would not only strengthen the current hypothesis but would be beneficial to understanding habituation at large, a process that despite its ubiquity is not well understood. This experiment has provided behavioural support for the hypothesis but cannot of course directly answer any questions concerning underlying mechanisms. However, knowledge of the possible functions of *avr-14* and the nature of synaptic transmission in *C. elegans* allows for some reasonable speculation.

There are several important considerations that need to be taken into account before making predictions. First, as mentioned earlier, the tap-withdrawal response is thought to be driven electrically by gap junctions while modulation of the response is thought to occur chemically (Wicks & Rankin, 1997). Inspection of the TWR circuit (see Figure 2) indicates that the chemical synapses made by sensory neurons of the two sub-circuits are onto components of the opposite circuit. ALM and AVM (sensory neurons of the head-touch circuit) make chemical synapses with AVB and PVC, both of which act as interneurons in the tail-touch circuit. PLM (sensory neuron of the tail-touch circuit) makes chemical synapses with AVD and AVA, both of which act as interneurons in the head-touch circuit. Based on

their work on the touch circuit, Chalfie and colleagues (1985) hypothesized that the chemical connections between sensory and interneurons in the touch circuit were functionally inhibitory. They suggested that head-touch and tail-touch lead, due to the connectivity of neurons in the circuit, to simultaneous activation of forward and backward components. Specifically, anterior touch lead to activation of interneurons, and subsequently motorneurons of the reversal circuit, as well as simultaneous inhibition of the competing, inappropriate forward response. This functional inhibition of the inappropriate response was said to occur via the chemical synapses between sensory neurons mediating anterior touch and the interneurons of the opposing circuit (Chalie et al., 1985). There is a large degree of overlap between neurons in the touch circuit and the TWR circuit, and many of the synapses are shared by the two circuits (Wicks & Rankin, 1995). This suggests a similar function for these synapses. There is some evidence to suggest that the chemical synapses between sensory and interneurons of the TWR circuit are also functionally inhibitory. As part of their effort to identify neurons involved in the TWR circuit Wicks and Rankin (1995) quantified the effects of ablating individual neurons thought to be part of the TWR circuit on both reversals and accelerations. The functional outcomes of several ablations support the hypothesis of functional inhibition. AVM ablations resulted in animals that reversed much smaller distances. This ablation resulted in both a loss of gap junction input to AVD and ALM, but also a loss of presumably inhibitory input to AVB and PVC (interneurons of the tail-touch circuit). ALM ablations resulted in worms that predominantly accelerated in response to tap. This may have been due to a loss of gap junction input to AVD, but also may have been due, as in the case of AVM, to an absence of inhibitory input to PVC. With no inhibition of the competing tail-touch circuit worms reversed less or not at all. Interestingly, ablation of both ALM and PVC resulted in a significantly higher frequency of reversals to tap. Removal of

PVC effectively prevented the absence of inhibitory input occurring in ALM and AVM ablated worms from exerting its effect on the tap-withdrawal response. Ablations of both ALM and AVM simultaneously resulted in animals that always accelerated to tap, similar to ablations of ALM alone. However an analysis of the magnitude of acceleration showed that accelerations in ALM,AVM ablated animals were more vigorous than in ALM ablated animals, suggesting that the connections between AVM and the PVC and AVB interneurons are in fact inhibitory, as the absence of inhibitory input lead to more pronounced accelerations. Ablations of PLM neurons resulted in animals that always reversed to tap, due to the loss of gap junction input to PVC, but the reversals in PLM ablated worms were larger than in non-ablated control worms. This suggests that the absence of the functionally inhibitory synapses between PLM and AVD and AVA resulted in a loss of inhibition on the head-touch circuit, leading to larger reversal magnitudes. More specifically, it was ablations of the right PLM neuron, but not the left PLM neuron that resulted in these effects. It is the right PLM neuron that makes chemical connections. Another line of evidence comes from a study investigating the functional connectivity of neurons in the TWR circuit using a computer simulation (Wicks, Roehrig, & Rankin, 1996). All possible polarity combinations could be assessed using the simulation and the computer generated output for each combination was compared to behavioural results of ablation experiments to determine which combination of polarities in the TWR circuit resulted in the best fit. It was concluded from this experiment that all five sensory neurons are inhibitory.

The second consideration is that in *C. elegans* there are no classic sodium-dependant action potentials, synaptic transmission is graded, and there is tonic release of neurotransmitter (Miriam, Hall, Avery, & Lockery, 1998; Davis & Stretton, 1989). The postsynaptic effects of communication through synapses with these properties have been shown to depend on the

functional nature of the cells involved. In excitatory motorneurons presynaptic injection of increasing depolarizing current results in graded increases in depolarizing postsynaptic muscle responses. Similarly, presynaptic injection of increasing hyperpolarzing current results in a graded increase in hyperpolarizing postsynaptic muscle response (Davis & Stretton, 1989). On the other hand, in inhibitory motorneurons the opposite is seen. Presynaptic injection of increasing depolarizing current results in graded increases in hyperpolarizing postsynaptic muscle responses, and presynaptic injection of increasing hyperpolarizing current results in graded increases in depolarizing postsynaptic muscle responses (Davis & Stretton, 1989). Thus, depending on whether the presynaptic neuron is functionally inhibitory or excitatory, the postsynaptic effects are strongly influenced.

A case has been made that the sensory neurons of the TWR circuit are functionally inhibitory (Wicks & Rankin, 1995; Wicks et al., 1996). In wild-type worms it is hypothesized that *avr-14* is an inhibitory autoreceptor on the sensory neurons of the TWR circuit. Repeated tap stimuli activate and depolarizes the sensory neurons causing glutamate release that then activates the *avr-14* inhibitory autoreceptor which would hyperpolarize the cell. This might lead to lower levels of neurotransmitter release and thus increased depolarization of the interneurons. In *avr-14* worms repeated tap stimuli may lead to larger or longer depolarization of the sensory neurons, due to the absence of the inhibitory *avr-14* autoreceptor. This might lead to increased levels of neurotransmitter release and thus increased hyperpolarization of the interneurons. The presence of *avr-14* on the sensory neuron may thus decrease the overall excitability of the sensory neurons. However, this is an oversimplified explanation that does not take two important factors into account. First, the precise expression pattern of *avr-14* limits hypotheses that can be made. Dent and colleagues (2000) stated that *avr-14* was expressed on PLM, PVD and ALM, and the following discussion is based on the assumption

that this is true. Second, it does not take into account the role of the competing forward and backward sub-circuits, both of which are simultaneously activated by the tap stimulus. Though the behavior of interest in this experiment was reversal in response to tap, and so will be the focus of this discussion, the behavior is the result of integrated output from two competing sub-circuits. An examination of the role of *avr-14* in each sub-circuit independently is useful before attempting to grasp the effects of *avr-14* on the kinetics of the entire circuit. In the reversal circuit, the absence of *avr-14* on PLM (tail touch sensory neurons) and PVD (putative stretch receptor involved in both forward and backward movement) is expected to result in higher levels of depolarization in these neurons with repeated stimulation. Higher levels of depolarization in PLM and PVC are expected to result in higher levels of transmitter release and increased levels of hyperpolarization in interneuron AVD (and subsequently AVA). This in turn may affect depolarizing gap junction input from ALM and AVM (head touch sensory neurons) to interneuron AVD (and subsequently interneuron AVA) with repeated stimulus presentation. Specifically, subsequent gap junction activation of the reversal circuit by ALM and AVM may be compromised by the fact that the command interneurons are in a hyperpolarized state. This would result in smaller reversal magnitudes with repeated stimulation. Gap junction input from ALM may actually be somewhat higher in the absence of *avr-14* than in its presence. The absence of *avr-14* on ALM is expected to lead to larger or longer depolarization, which on its own should lead to larger depolarizations of interneuron AVD (and subsequently interneuron AVA) and consequently larger reversal magnitudes. However, given the presumed hyperpolarization of the command interneurons due to chemical synaptic input from three sensory neurons (1 PLM and 2 PVCs), the increased gap junction input due to ALM may not be large enough to significantly affect behavior. In wild-type worms on the other hand, repeated tap presentation

is expected to result in hyperpolarization of PLM and PVD due to the action of the inhibitory *avr-14* autoreceptor. This hyperpolarization is expected to cause lower levels of transmitter release from the sensory neurons and depolarization in the AVD interneuron (and subsequently interneuron AVA). Depolarizing input to AVD (and its follower AVA) via gap junction input from ALM and AVM may thus not be compromised to the same extent as in the absence of *avr-14*. On the contrary, in wild-type worms depolarizing gap junction input from ALM and AVM may lead to larger reversal magnitudes since due to the previous tap stimulus the interneurons are already in a state of increased excitability. The combined effect in wild-type worms should be larger reversal responses (slower habituation) with repeated tap stimuli relative to *avr-14* worms. In the forward circuit that mediates accelerations, the absence of *avr-14* on the PLM sensory neurons is expected to result in higher levels of depolarization in those neurons. This may result in higher levels of depolarization of PVC interneurons (and subsequently interneurons AVB) via gap junction input from PLM, resulting in higher levels of activation of the competing forward response. This increase in "interference" by the competing forward circuit in theory should decrease both the magnitude and frequency of reversals with repeated taps. In *avr-14* worms the absence of the inhibitory autoreceptor on PVD will lead to increased depolarization of PVD with repeated tap presentation and increased levels of transmitter release. This should lead to increased hyperpolarization of PVC interneurons (and subsequently AVB interneurons), resulting in higher levels of inhibition of the forward response. The absence of the *avr-14* receptor on ALM is also expected to result in increased depolarization of that sensory neuron, which should result in higher levels of transmitter release and hyperpolarization in interneuron PVC. However at the same time, with repeated stimulation the forward circuit is receiving depolarizing chemical synaptic input from AVM, which itself is becoming hyperpolarized with repeated tap

presentation. This should result in more activation of the forward response. However, that depolarizing gap junction input to interneuron PVC is clearly compromised by the increased hyperpolarizing chemical synaptic input to PVC from PVD. Thus, in *avr-14* worms the effects on the forward movement circuit are mixed. On the one hand, there should be higher levels of activation of the forward circuit, but at the same time there are other factors which compromise the increase in activation and lead to increased inhibition of the competing forward circuit. The net gain of these two opposing factors is hypothesized to be increased activation of the forward circuit in *avr-14* worms compared to wild-type worms. In wild-type worms on the other hand, with repeated tap stimuli, PLM is expected to become hyperpolarized due to the inhibitory action of *avr-14* receptor. This would cause less activation of the forward response via gap junction input from PLM to interneuron PVC (and subsequently AVB) and thus should not interfere with the competing reversal response to the same extent as in *avr-14* worms. PVD is also expected to become hyperpolarized with repeated tap presentation which should lead to lower levels of transmitter release and increased depolarization of PVC interneurons (and subsequently AVB interneurons) resulting in larger activation of the forward response. Similarly, ALM is expected to become hyperpolarized with repeated stimulation. This should however result in lower levels of transmitter release and higher levels of depolarization in PVC (and subsequently AVB) resulting in higher levels of activation of the forward response. Finally, AVM is expected to become depolarized with repeated stimulation which should cause increased transmitter release and increased hyperpolarization of PVC and AVB, resulting in increased inhibition of the forward response. In summary, in wild-type worms overall there seems to be some activation of the competing forward circuit with repeated tap stimuli. This may contribute to the reversal magnitude decrease observed with repeated stimulation. What distinguishes *avr-*

14 worms from wild-type worms may be the increased levels of hyperpolarization in interneurons AVD and AVA. The resulting effects on reversal magnitudes may be a reason rates of habituation observed in *avr-14* worms are more rapid than in wild-type worms. The decreases in variability seen at the 10s ISI may also be a result of differences in excitability of the sensory neuron. Perhaps, in wild-type worms the magnitude or duration of depolarization of the sensory neurons may be compromised by simultaneous hyperpolarization due the action of *avr-14*. The exact level of depolarization in the wild-type sensory neuron may vary from stimulus to stimulus, resulting in larger variability of reversal magnitudes. In *avr-14* worms on the other hand, the sensory neurons are consistently being depolarized with repeated stimulation which may lead to consistently lower activation of the reversal circuit, resulting in smaller less variable reversal magnitudes.

The hypotheses made can be tested using laser ablation of specific sensory neurons. If this scenario is correct then ablating PLM or PVD neurons in *avr-14* worms should attenuate the increased rates of habituation at a 10s ISI, specifically because this manipulation would effectively remove the hyperpolarization of interneurons AVD and AVA by the PLM and PVD sensory neurons. On the other hand, ablating ALM would have a lesser impact; it would remove the increases in activation of the competing forward response, but the hyperpolarization of AVD and AVA from PLM and PVD would be unchanged leading to decreased reversal magnitudes with repeated stimulation as seen in Experiment 1.

It is much more difficult to account for the finding that the rate of habituation in *avr-14* was only altered at the 10s ISI. Presumably, the action of *avr-14* in influencing sensory neuron excitability should be the same regardless of stimulation protocol. The account given above is most readily applied to the behavioural data seen for habituation with a 10s ISIs in *avr-14*, but cannot account for the apparent lack of differences in habituation seen at 45s and

60s ISIs. One potential explanation is that the effects of *avr-14* on excitability of the sensory neurons are time dependent. At longer ISIs, in *avr-14* worms excitability of the sensory neuron may have returned to baseline levels. The subsequent effects on the interneurons may also be time-dependant processes. For example the described hyperpolarization of AVD and AVA in *avr-14* worms may only last a certain period of time. At long ISIs excitability of interneurons may have returned to baseline levels and subsequent depolarizing input may not be compromised to the same extent.

An implicit assumption here is that the effects of *avr-14* activation on sensory neuron excitability and subsequent glutamate release (and consequent activity in the interneurons) are direct. Though this assumption is not unreasonable, given that *avr-14* activation results in increased passage of chloride ions into to cell (Dent et al., 2000), there is no reason to rule out the possibility of intervening steps. In theory at least, there could be any number of intervening steps occurring between *avr-14* activation and transmitter release. One or more of these could affect transmitter release and could also be sensitive to the ISI used during training.

Experiment 2: Long-term Memory for Distributed and Massed Training at a 60 Second ISI in *avr-14*

To assess the role of *avr-14* in long-term (>24 hours) memory, worms were run using a distributed and a massed training protocol. *avr-14* appears to be either directly or indirectly involved in glutamate release, and the importance of presynaptic glutamate release in producing long-term memory for habituation in *C. elegans* has previously been shown (Rose et al., 2002). Thus, it was hypothesized that *avr-14* mutations may also have an effect on long-term memory for habituation.

Method

Subjects A total of 48 *avr-14* (da1371) and 48 N2 worms (24 per group) were used in the distributed training experiment, and a total of 40 *avr-14* (da1371) and 40 N2 worms (20 per group) were used in the massed training experiment.

Procedure Four groups of worms were run at one time in both the distributed and the massed training experiments: *avr-14* trained, *avr-14* control, N2 trained, N2 control. Approximately 15- 20 worms were pre-plated 18-20 hours before the beginning of training on plates streaked with *E. coli*. In the distributed training experiment, worms in the distributed training group received four blocks of twenty taps at a 60s ISI separated by 1-hour rest intervals. Worms in the control group received only a single tap on training day. At least one hour after the last training block, worms from all four groups were transferred onto individual labeled *E. coli* streaked plates. Worms were kept in cushioned boxes for the period between transfer and testing. Testing occurred 22-28 hours following the end of training with the median retention test interval being greater than 24 hours. Worms from all groups were tested individually with 10 taps presented at a 60s ISI. Only responses during the testing phase of the experiment were videotaped, scored, and measured. In the massed training experiment, worms in the massed training group received eighty taps at a 60s ISI in one continuous block without any rest periods. Worms in the control group received only a single tap on training day. Transfer, overnight storage, and testing for worms in the massed training experiment occurred in the same way as described above for the distributed training experiment.

Statistical Analysis Significantly smaller mean response magnitudes in trained compared to control worms were taken as evidence for long-term memory. Mean reversal magnitudes for each worm across the series of ten test stimuli were computed. For each strain, data from the distributed or massed training group were standardized relative to their respective controls.

Two separate two-way between subjects ANOVAs on the mean standardized reversal magnitudes were conducted for the massed and distributed training experiments respectively, with two levels of the strain factor (*avr-14* and N2) and two levels of the group factor (trained and control).

Results and Discussion

Mean standardized reversal magnitudes for distributed training are shown in Figure 10. Both *avr-14* and N2 worms showed significantly lower mean response magnitudes as a result of distributed training. ANOVA revealed only a significant effect of group (F (1, 92) = 14.021, $p < 0.001$) indicating that overall mean reversal magnitudes were significantly lower in trained compared to control worms regardless of strain. There was no evidence to indicate that there was any difference between the two strains in the size of the response decrement produced by distributed training. A separate planned comparison between trained *avr-14* and trained N2 worms using Fisher's least significant differences planned comparison method supported this ($p > 0.05$; see Figure 10). Mean standardized reversal magnitudes for massed training are shown in Figure 11. Neither strain showed evidence of retention for massed training. Statistical analyses supported this conclusion. ANOVA revealed no significant main effects (F (1, 76) = 0.53, $p > 0.1$ and F (1, 76) = 2.688, $p > 0.1$ for strain and group respectively) or any significant interaction between the two factors (F (1, 76) = 0.053, $p > 0.1$). The results from Experiment 1 and the current experiment suggest some possible parallels between processes underlying short-term memory and processes underlying long-term memory. The *avr-14* mutation did not affect short-term habituation at a 60s ISI, nor based on Experiment 2, did it appear to affect long-term habituation at a 60s ISI. This also suggests that the *avr-14* mutation may not affect the transition from short-term to long-term memory. This supports the notion that the effects of *avr-14* on glutamate release are likely

Figure 10. Mean reversal magnitudes (± SEM) in *avr-14* and N2 worms that received distributed training at a 60s ISI (Trained, n=24 per strain) or a single tap (Control, n=24 per strain). Mean reversal magnitudes were expressed as a percentage of the control group response. Both *avr-14* and N2 worms showed significantly smaller responses as a result of distributed training at a 60s ISI compared to their respective single-tap matched controls (** $p < 0.001$).

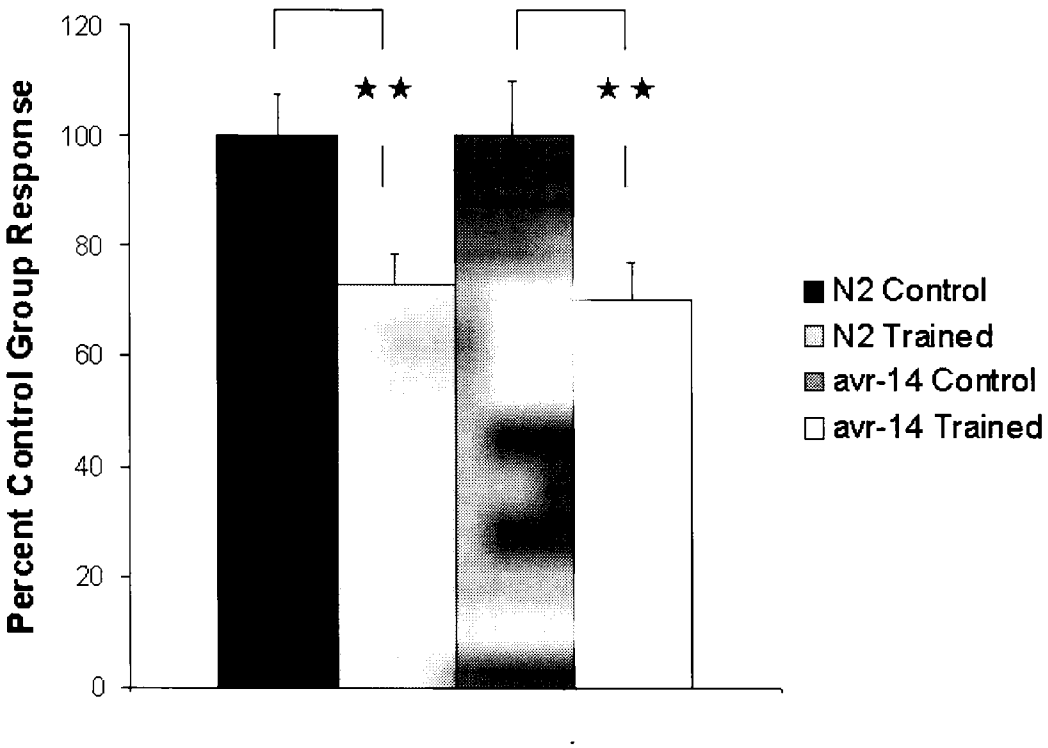
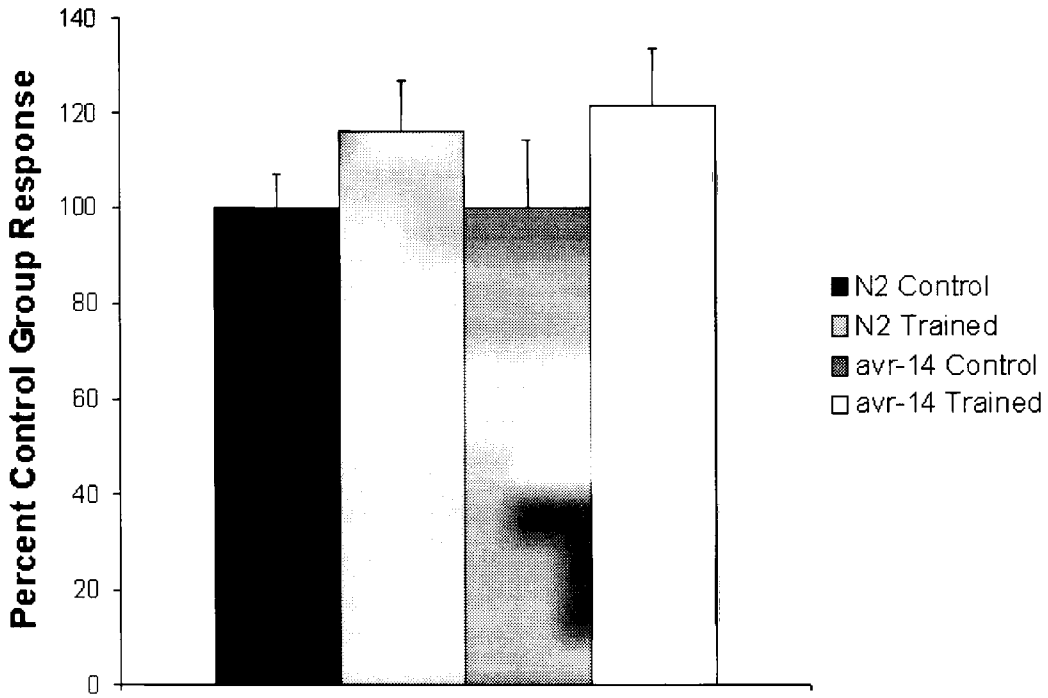

Percent Control Group Response
120
100
80
60
40
20
0
★★
★★
N2 Control
N2 Trained
avr-14 Control
avr-14 Trained

Figure 11. Mean reversal magnitudes (± SEM) in *avr-14* and N2 worms that received massed training at a 60s ISI (Trained, n=20 per strain) or a single tap (Control, n=20 per strain). Mean reversal magnitudes were expressed as a percentage of the control group response. Neither *avr-14* nor N2 worms showed significantly smaller responses as a result of massed training at a 60s ISI compared to their respective single-tap matched controls.


Percent Control Group Response
140
120
100
80
60
40
20
0
N2 Control
N2 Trained
avr-14 Control
avr-14 Trained

indirect and ISI-dependant and/or time-limited. If the absence of the *avr-14* receptor on the sensory neuron lead to generalized increases in glutamate transmission across ISIs then differences in long-term habituation with a 60s ISI would be expected. Perhaps this difference would be reflected as enhanced retention for distributed training, as indicated by larger mean response decrements. However, this was clearly not the case in Experiment 2.

Neither strain showed any evidence for retention of massed training when tested at 24 hours. It needs to be noted that wild-type worms do show significant retention for massed training at 12 hours post training (Rose & Rankin, 2002a). Thus, the choice of 24 hours for testing memory for massed training in *avr-14* worms may have been an incorrect one. If *avr-14* worms do in fact show any differences in retention for massed training at a 60s ISI, then these differences are more likely to be observed at 12 than at 24 hours. This possibility will certainly be examined in future experiments.

Data from Experiment 2 suggest that there are similarities in long-term memory for habituation at a 60s ISI. Both strains require distributed training in order to show significant long-term memory. In this regard *avr-14* worms appear to be no different than other *C. elegans* strains or many other species tested (Quinn & Dudai, 1976; Carew et al., 1972; Mauelshaugen et al., 1998; Ebbinghaus, 1885/1964).

Experiment 3: Effects of Heat Shock on Long-term Memory for Distributed Training at a 60 Second ISI in *avr-14*

In wild-type worms, long-term memory for habituation when trained at a 60s ISI is known to depend on protein synthesis (Beck and Rankin, 1997; Rose et al., 2002). This experiment sought to assess whether long-term memory for the same training protocol in *avr-14* shared this property.

Methods

Subjects A total of 80 *avr-14* (da1371) worms and 80 N2 worms were used in this experiment. There were 20 worms in the distributed training group and 20 worms in the tap control group for each of the heat-shock and sham-shock conditions in each of the two strains.

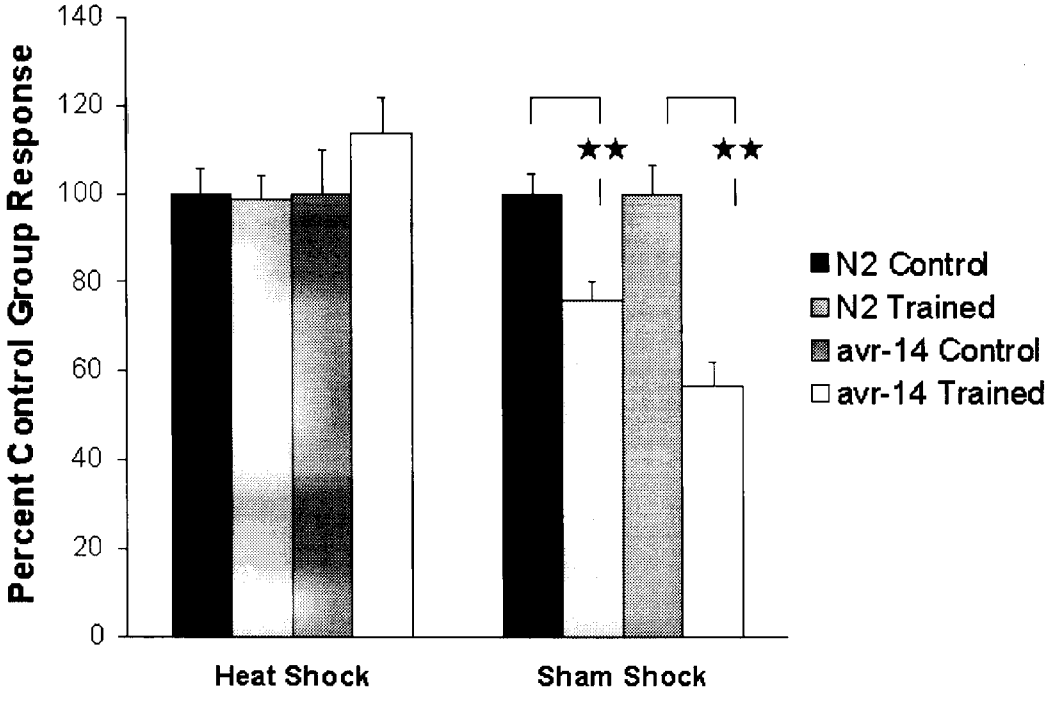
Procedure The same distributed training protocol as in Experiment 2 was used. Immediately after the last tap stimulus within each training block, both the trained group and the control group were either heat-shocked or sham-shocked. Heat shock was given by double-wrapping both trained and control groups with parafilm immediately after the last tap stimulus within each training block and submerging them in a 32 °C water bath for the first 40 minutes of every 1-hour rest period. For the remainder of the rest period, plates were returned to room temperature. 32 °C was chosen because previous research has shown that heat shock proteins are activated at this temperature in *C. elegans* (Beck & Rankin, 1997). Since there was a fair bit of handling involved in the heat shock procedure, a sham shock group was run as well to control for any effects this may have had. Sham shock was given in the same manner as heat-shock, except that plates were submerged in a 21 °C water bath.

Statistical Analysis Data was standardized as in Experiment 2. A three-way between subjects ANOVA was conducted on the mean standardized reversal magnitudes, with two levels of the strain factor (*avr-14* and N2), two levels of the condition factor (heat shock and sham shock) and two levels of the group factor (trained and control). Where appropriate significant interactions were further analyzed.

Results and Discussion

Figure 12 shows mean standardized reversal magnitudes in both strains across the various groups and conditions. In both strains, administration of 40 minutes of heat shock during the 1-hour rest intervals blocked the formation of long-term memory. Administration

Figure 12. Mean reversal magnitudes (± SEM) expressed as a percentage of the single-tap matched control group response in worms given heat shock (HS; a 32 °C water bath) or sham shock (SS; a 21 °C water bath) for the first 40 min of every 1-hour rest period in the 60s ISI distributed training protocol. Mean reversal magnitudes in groups of either strain that received distributed training with heat shock (HS Trained, n=20 per group) did not differ from mean reversal magnitudes in single-tap matched controls that also received heat shock (HS Control, n=20 per group). Mean reversal magnitudes in groups of either strain that received distributed training with sham shock (SS Trained, n=20 per group) were significantly smaller than mean reversal magnitudes in single-tap matched controls that also received sham shock (SS Control, n=20 per group) (** $p < 0.001$).


Percent Control Group Response
140
120
100
80
60
40
20
0
Heat Shock
Sham Shock
★★
★★
N2 Control
N2 Trained
avr-14 Control
avr-14 Trained

of sham-shock on the other hand did not block the formation of long-term memory in either strain. Statistical analysis supported these conclusions. An ANOVA revealed a significant group by condition interaction (F (1, 152) = 19.766, $p < 0.001$). Subsequent simple main effects analysis revealed a significant main effect of group (control vs. distributed) in the sham shock condition (F (1, 152) = 14.085, $p < 0.001$) but not in the heat shock condition (F (1, 152) = 0.480, $p > 0.05$), indicating that heat shock did in fact block formation of long-term memory regardless of strain.

Closer inspection of Figure 12 suggests that the response decrement observed in sham-shocked trained *avr-14* worms is larger than that seen in sham-shocked trained N2 worms compared to their respective controls (roughly 47% in *avr-14* vs. 24% in N2 worms). Even though this difference is not large enough to warrant a statistically significant three-way interaction, a separate planned comparison using Fisher's least significant difference planned comparison method revealed that the mean reversal magnitudes in trained *avr-14* worms exposed to sham shock were significantly smaller than mean reversal magnitudes in trained wild-type worms exposed to sham shock ($p < 0.05$; see Figure 12). This seems at odds withthe results of Experiment 2, which showed no difference in the amount of retention between the two strains (cf. Figure 12 and Figure 10). The reason for this is not understood. It seems unlikely that the sham shock procedure was the cause of the difference. The temperature of the sham shock water bath was held constant at 21 °C, which was comparable to the room temperature conditions that *avr-14* worms in Experiment 2 were kept at during the 1-hour rest intervals. Great care was taken to handle plates very gently, so it also seems unlikely that *avr-14* worms in the distributed training condition receiving sham shock had received higher levels of stimulation due to handling. The results from Experiment 2 were in fact not in agreement with previous pilot data, which have suggested a retention enhancement

in *avr-14* worms for distributed training with a 60s ISI (Rose & Rankin, personal communication). It is thus not completely clear whether or not *avr-14* worms show an enhancement in long-term memory for this training protocol. This uncertainty complicates any hypotheses of the effects of *avr-14* on sensory neuron excitability and glutamate release. Though it can be said with some certainty that the absence of *avr-14* does not appear to make a difference for training with a 60s ISI in the short-term, the same conclusion can at this point not be made as firmly with regard to long-term memory.

Whether or not there exist any differences in the amount of retention for distributed training between the two strains, the results from this experiment show that long-term memory for distributed training with a 60s ISI in both strains shares a common dependence on protein synthesis. This suggests that the long-term memory in both strains also shares similar postsynaptic mechanisms important for the formation of long-term memory. What exactly these mechanisms are is currently being actively pursued. It is known that long-term memory requires intact *glr-1* receptors (40 % homology to mammalian AMPA-type receptors; Hart et al., 1995) on the interneurons of the TWR circuit. The distributed training protocol with a 60s ISI results in distinct quantifiable changes in postsynaptic *glr-1* receptors. Using a strain of worms expressing a GLR1:GFP (green fluorescent protein) genetic construct, Rose and Rankin (2002b) demonstrated that four blocks of distributed training with a 60s ISI resulted in the same number of GLR-1:GFP clusters on postsynaptic neurons as in control worms but less GFP per cluster. This suggested that the LTH protocol resulted in a down-regulation of postsynaptic *glr-1* receptors. This down-regulation of *glr-1* was specifically blocked by heat shock suggesting a dependence on protein synthesis. Taken together, these results strongly suggest that long-term memory for distributed training at a 60s ISI is critically dependent on postsynaptic changes, and that specifically *glr-1* down-regulation is a crucial factor for the

behavioural expression of long-term memory for distributed training in *C. elegans* (Rose & Rankin, 2002b). As long-term memory for distributed training in *avr-14* worms was also shown to depend on protein synthesis it seems likely that the same postsynaptic down-regulation of *glr-1* receptors is occurring.

Experiment 4: Long-term Memory for Distributed and Massed Training at a 10 Second ISI in *avr-14*

In wild-type worms a 60s ISI during distributed training leads to long-term memory, while a 10s ISI during distributed training does not, suggesting that the cellular mechanisms recruited during habituation to long ISIs support a transition to long-term memory whereas cellular mechanisms recruited during habituation to short ISIs do not. An alternative explanation may be that in wild-type worms short ISIs recruit one or more additional mechanisms that block transition to long-term memory. The same processes may be recruited during training with long ISIs, but additional processes also recruited during training with long ISIs may ultimately support a transition to long-term memory. If this were in fact the case, then a worm defective in the hypothesized mechanism blocking the transition to long-term memory, would be expected to show evidence of long-term memory when trained with short ISIs. In addition, it could also be predicted that such a worm may show enhanced long-term memory when trained with long ISIs, provided that long ISIs do in fact recruit this same blocking process. Experiments 2 and 3 offered mixed results about the possibility of enhanced memory in *avr-14* worms when trained with long ISIs. The possibility remains that worms defective in such a mechanism may show evidence of long-term memory when trained with short ISIs. *avr-14* seems a reasonable candidate in which to test this hypothesis. Experiment 1 clearly showed a difference in short-term habituation at 10s ISIs in this strain. It was hypothesized that since *avr-14* was involved in a cellular mechanism unique to short ISIs, it

might also be involved in the transition from short to long-term memory when trained with short ISIs.

Method

Subjects A total of 72 *avr-14* (da1371) and 72 N2 worms (36 per group) were used in the distributed training experiment and a total of 66 *avr-14* (da1371) and 66 N2 worms (33 per group) were used in the massed training experiment.

Procedure The same procedure was used as in Experiment 2 with the one difference that all training and testing was done using a 10s ISI. Thus, worms in the distributed training groups were given four blocks of twenty taps each at a10s ISI separated by 1-hour rest intervals and worms in the massed training groups were given eighty taps at a 10s ISI in one continuous block.

Statistical analysis The same analysis as in Experiment 2 was employed. Where appropriate, significant interactions were further analyzed.

Results and Discussion

Mean standardized reversal magnitudes for distributed training at a 10s ISI are shown in Figure 13. Unlike wild-type worms, *avr-14* worms showed significantly lower mean response magnitudes as a result of distributed training at a 10s ISI. Statistical analyses supported these conclusions. An ANOVA revealed a significant interaction between strain and group ($F(1, 140) = 9.304$, $p < 0.01$). Simple main effects analysis of the group factor (trained vs. control) at the levels of the strain factor (*avr-14* vs. N2) revealed a significant simple main effect for group at *avr-14* ($F(1, 140) = 146.67$, $p < 0.001$), but not at N2 ($F(1, 140) = 0.235$, $p > 0.1$). Thus mean standardized response magnitudes in *avr-14* worms were significantly lower in trained compared to control worms ($p < 0.001$).

Figure 13. Mean reversal magnitudes (± SEM) in *avr-14* and N2 worms that received distributed training at a 10s ISI (Trained, n=36 per group) or a single tap (Control, n=36 per group). Mean reversal magnitudes were expressed as a percentage of the control group response. *avr-14* worms, unlike wild-type worms, that received distributed training at a 10s ISI showed significantly smaller responses compared to single-tap matched controls (** $p < 0.001$).

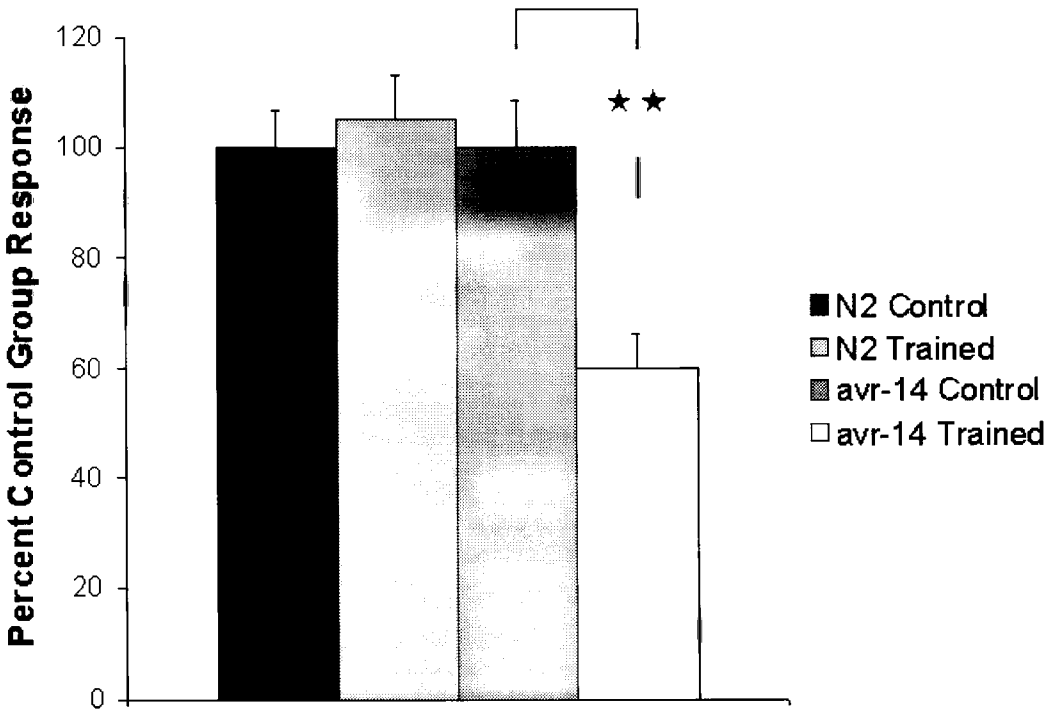
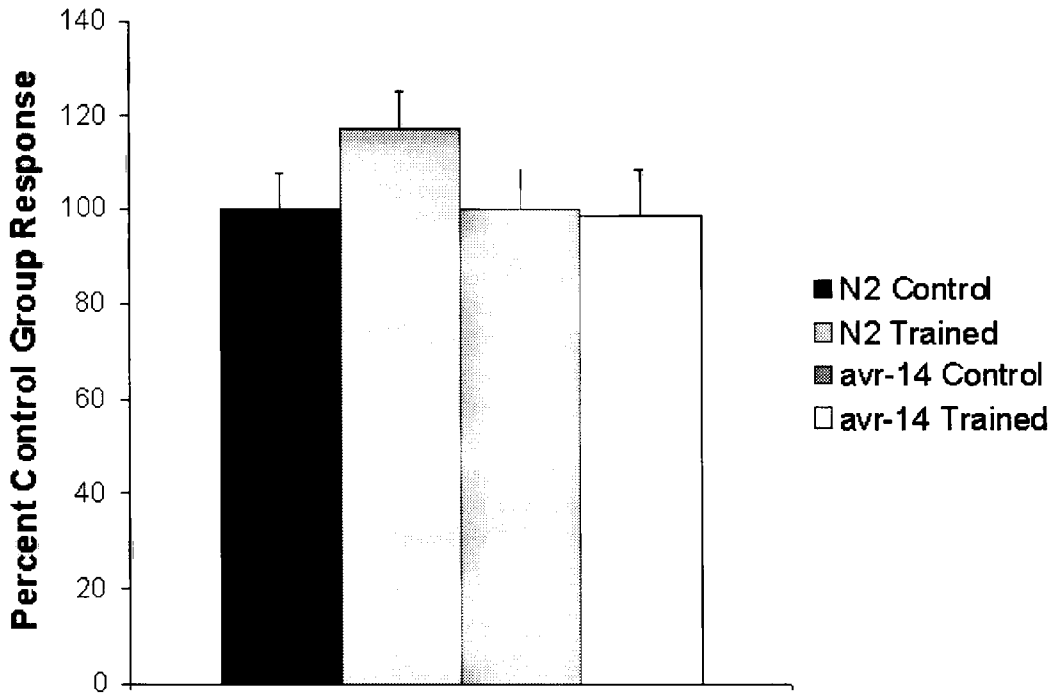

Percent Control Group Response
120
100
80
60
40
20
0
★★
N2 Control
N2 Trained
avr-14 Control
avr-14 Trained

Figure 14. Mean reversal magnitudes (± SEM) in *avr-14* and N2 worms that received massed training at a 10s ISI (Trained, n=33 per group) or a single tap (Control, n=33 per group). Mean reversal magnitudes were expressed as a percentage of the control group response. Neither *avr-14* worms nor N2 worms that received massed training at a 10s ISI showed significantly smaller responses compared to single-tap matched control worms.


Percent Control Group Response
140
120
100
80
60
40
20
0
N2 Control
N2 Trained
avr-14 Control
avr-14 Trained

Mean standardized reversal magnitudes for massed training at a 10s ISI are shown in Figure 14. Neither strain showed evidence for retention of massed training. Statistical analyses supported this conclusion. ANOVA revealed no significant main effects ($\underline{F}$ (1, 128) = 1.129, $\underline{p} > 0.1$ and $\underline{F}$ (1, 128) = 0.851, $\underline{p} > 0.1$ for strain and group respectively) or any significant interaction between the two factors ($\underline{F}$ (1, 128) = 1.129, $\underline{p} > 0.1$).

It was suggested that there may be a process unique to short ISIs, one that becomes active when the worm is trained with a 10s ISI, which specifically blocks a transition to a longer lasting form of memory. Removal of such a block should then allow for the formation of long-term memory when trained with short ISIs. It appears that in wild-type worms, *avr-14* may be part of such a process. In the presence of functional *avr-14* receptors, there is no evidence of long-term memory for training with short ISIs, while in its absence long-term memory for the same training was reliably obtained. This is a very unique and important finding for several reasons. First, it further supports an already established importance of *avr-14* in memory for habituation in *C. elegans*. Second, there is currently no other known example in the literature of a gene (and presumably its associated processes) whose presence blocks long-term memory and whose absence actually enhances long-term memory. Third, results provide additional support for the already established importance of presynaptic glutamate release in the formation of long-term memory in *C. elegans* (Rankin & Wicks, 2000; Rose et al., 2001; Rose and Rankin, 2002b). How similar is long-term memory observed in *avr-14* with short ISIs to that seen with long ISIs in wild-type worms? One aspect they certainly share in common is a dependence on distributed training. As with the 60s ISI, *avr-14* mutants only showed long-term memory when the stimuli were presented in blocks with 1-hour rest periods in between. Massed training with a 10s ISI clearly did not produce any long-term memory. However, again as in Experiment 2, differences in memory for

massed training with a 10s ISI may have been more appropriately investigated at 12 hours than at 24 hours (Rose and Rankin, 2002a). The necessity of distributed training blocks suggests a possible role for protein synthesis. This possibility was further investigated in Experiment 4.

It should be becoming increasingly apparent, that understanding the mechanisms through which *avr-14* works is crucial for an understanding of the cellular basis of learning and memory in *C. elegans*. Given the suggested role of *avr-14* as an autoreceptor and the unique properties of *C. elegans* neurons and synaptic communication, some reasonable speculation can be made. Though the mutation is presynaptic in nature, there are a number of effects the mutation may have on postsynaptic processes. This is an important consideration, especially in the case of long-term memory which in *C. elegans* is known to rely on postsynaptic mechanisms (Rose et al., 2002; Rose & Rankin, 2002b). A possible explanation is that the absence of *avr-14* on the sensory neuron results in no inhibition of glutamate release with repeated stimulation. The depolarization of the sensory neurons and the resulting graded increases in glutamate release with repeated high frequency stimulation in *avr-14* worms, that do not occur to the same extent in wild-type worms under similar conditions, may be causing the apparent "enhancement" in long-term memory seen with 10s ISI training. The fact that such an enhancement was only seen when trained with short ISIs but not when trained with long ISIs complicates the actuality of such a hypothesis. If the only difference between wild-type and *avr-14* worms were in fact higher levels of glutamate release with LTH training then these should occur regardless of ISI. This may be the case, with the one difference that the build-up of glutamate in the synaptic cleft is more rapid at a 10s ISI. The rapid build-up may be causing the same protein synthesis dependent postsynaptic changes that usually occur with distributed training at a 60s ISI (Rose & Rankin, 2002b). An easily testable prediction made

by such a hypothesis is that long-term memory for distributed training with a 10s ISI can be blocked by heat-shock.

Experiment 5: Effects of Heat Shock on Long-term Memory for Distributed Training at a 10 Second ISI in *avr-14*

Method

Subjects A total of 108 *avr-14* (da1371) worms were used in this experiment. 27 worms in the distributed training group and 27 worms in the tap control group for each of the heat shock and sham shock conditions.

Procedure The same procedure as in Experiment 3 was followed except of course that all training and testing was done at a 10s ISI.

Statistical Analysis Data was standardized as in Experiments 2, 3, and 4. A two-way between- subjects ANOVA was conducted on the mean standardized reversal magnitudes, with two levels of the condition factor (heat shock and sham shock) and two levels of the group factor (trained and control). Where appropriate significant interactions were further analyzed.

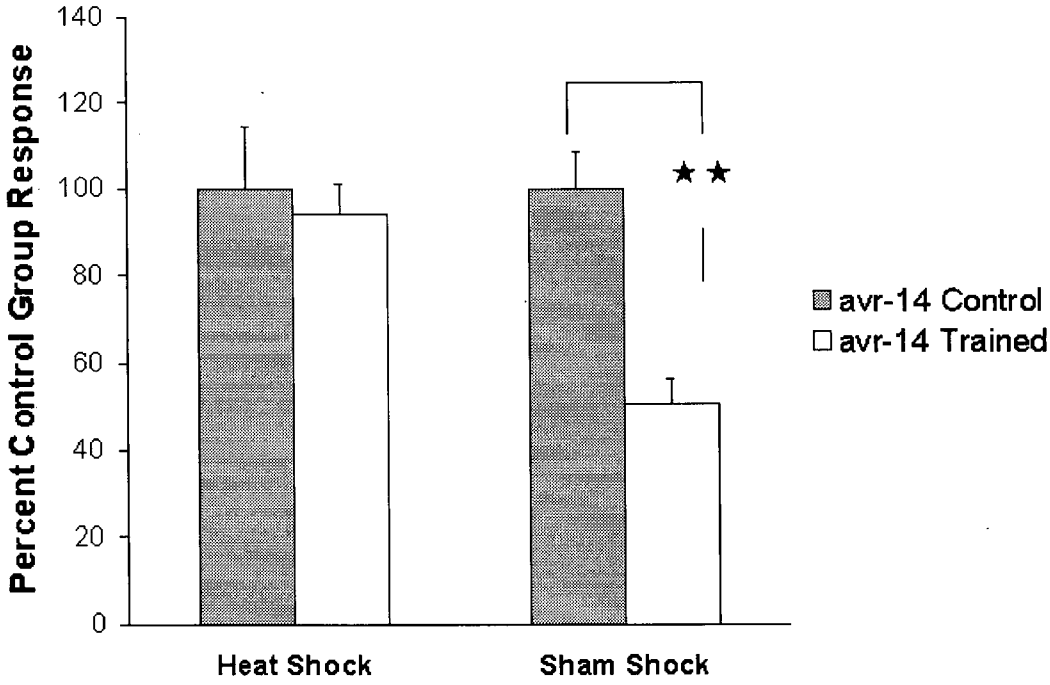
Results and Discussion

Mean standardized reversal magnitudes for distributed training at a 10s ISI in heat shocked and sham shocked *avr-14* worms are shown in Figure 15. Administering heat-shock for the first 40 minutes after each training block disrupted the formation of long-term memory when tested 24 hours later. On the other hand, administering sham shock for the first 40 minutes after each training block did not disrupt the formation of long-term memory. Statistical analysis supported these conclusions. An overall ANOVA on the four groups revealed a significant interaction between condition and group ($F(1, 104) = 5.342$, $p < 0.05$). Simple main effects analysis of the group factor (trained vs. control) at the levels of the condition

factor (heat-shock vs. sham-shock) revealed a significant simple main effect of group in the sham shock condition (F (1,104) = 13.657, $p < 0.001$), but not in the heat-shock condition (F (1, 104) = 0.183, $p > 0.1$). Thus, mean standardized response magnitudes were significantly lower in trained worms compared to single-tap controls only in worms that were exposed to sham shock ($p < 0.001$) and not in worms that were exposed to heat shock ($p > 0.05$).

One characteristic that long-term memory in *avr-14* for distributed training with a 10s ISI and long-term memory for distributed training with a 60s ISI share is a dependence on protein synthesis. This suggests that long-term memory for training with a 10s ISI in *avr-14* does in fact require the same postsynaptic mechanisms discussed earlier in connection with Experiments 2 and 3. Very likely, the same postsynaptic mechanisms are being engaged by the 10s ISI training protocol as by the 60s ISI training protocol in both wild-type and *avr-14* worms. Long-term memory for distributed training with a 10s ISI is expected to require postsynaptic *glr-1* receptors and the resulting changes in *glr-1* expression described by Rose and Rankin (2002b), and these changes would likely be blocked by administration of heat shock. The first hypothesis can be investigated fairly easily. In wild-type worms administration of DNQX, a non-selective AMPA/kainate receptor antagonist, during the LTH training protocol blocks the expression of long-term memory (Rose et al., 2001). Administration of DNQX during the 10s ISI training protocol in *avr-14* is expected to result in a similar block of long-term memory. The second hypothesis can be tested using a GLR-1:GFP construct in an *avr-14* background. That way, postsynaptic changes in *glr-1* expression in *avr-14* worms as a result of the 10s ISI training protocol could be quantified in the same manner as has been previously done.

Figure 15. Mean reversal magnitudes (± SEM) expressed as a percentage of the single-tap matched control group in *avr-14* worms given heat shock (HS; a 32 °C water bath) or sham shock (SS; a 21 °C water bath) for the first 40 min of every 1-hour rest period in the 10s ISI distributed training protocol. Mean reversal magnitudes in worms that received distributed training with heat shock (HS Trained, n=27) did not differ from mean reversal magnitudes in single-tap matched controls that also received heat shock (HS Control, n=27). Mean reversal magnitudes in groups that received distributed training with sham shock (SS Trained, n=27) were significantly smaller than mean reversal magnitudes in single-tap matched controls that also received sham shock (SS Control, n=27) (** $p < 0.001$).


Percent Control Group Response
140
120
100
80
60
40
20
0
Heat Shock
Sham Shock
★★
avr-14 Control
avr-14 Trained

One implication of this hypothesis is that the difference(s) between *avr-14* and wild-type worms that results in the formation of long-term memory when trained with a 10s ISI in one strain but not the other may be presynaptic, within the sensory neurons. Specifically the data from Experiment 4 suggest that the absence of *avr-14* on the sensory neuron removes some type of cellular block whose absence may allow for the formation of long-term memory. In wild-type worms on the other hand the presence of this cellular process may be what blocks the formation of long-term memory for training with a 10s ISI. As the exact function(s) of *avr-14* in the sensory neuron are not clearly understood it is also unclear what exactly this "block" is. However, it is reasonable to speculate that this cellular blocking process is influencing glutamate release and/or transmission. Once again, the absence of *avr-14* on the sensory neurons may results in greater excitability of the sensory neuron and greater graded increases in glutamate release. These may then in turn activate the postsynaptic changes described above. In wild-type worms at a 10s ISI, with repeated high-frequency stimulation the hyperpolarizing effect of the *avr-14* autoreceptor may lead to a decrease in excitability of the sensory neuron and thus smaller increases in glutamate release. Less glutamate in the synaptic cleft in wild-type worms compared to *avr-14* worms may not be sufficient to initiate these postsynaptic changes, at least not to a level that produces long-term memory.

General Discussion and Suggestions for the Future

The series of experiments presented in this paper has illustrated an important role for the *avr-14* gene in both short-term and long-term memory for habituation in *C. elegans*. The effects of mutations in *avr-14* are dependent on the ISI used during training. Experiment 1 showed that in short-term memory for habituation the effects are clearly strongest at the 10s ISI. Here habituation occurs more rapidly and asymptotic response levels are lower compared to wild-type worms. Results from the habituation experiments with the 30s ISI also indicate a

role for *avr-14* in habituation using these training parameters. There are no differences in the rate or development of habituation, but nevertheless asymptotic response levels were clearly lower compared to wild-type worms. At the long ISIs, 45s and 60s, there appear to be no differences in either the rate or the level of habituation between *avr-14* mutants and wild-type worms. As the effects of the *avr-14* mutation on spontaneous recovery from habituation with a 45s ISI do not seem reliable, it also appear that the effects on this process are confined to the 10s ISI. Thus, *avr-14* is the first example of a mutant that in *C. elegans* predominantly affects habituation to short ISIs. Based on these experiments short ISIs can be defined as ISIs less than 30s. The original hypothesis of multiple ISI-dependant short-term memory systems put forth by Rankin and Broster (1992) made the prediction that there should be specific genes that are differentially involved in habituation according to the ISI used during training. *avr-14* provides an additional piece of support for this hypothesis. There are also examples of genes that as suggested by the hypothesis are common to all ISIs. For example, short-term habituation in *eat-4* mutants occurs more rapidly and asymptotic response levels are lower at both 10s and 60s ISIs (Rankin & Wicks, 2000). *eat-4* encodes a vesicular glutamate transporter expressed on the sensory neuron of the TWR circuit (Lee et al., 1999). *eat-4* mutants show normal responses to tap but more rapid habituation to tap regardless of ISI. This gene may thus be an example of a process that is common to habituation at all ISIs. Based on the results of the current experiment a reasonable question to ask is how the hypothesized cellular processes are selectively recruited by different ISIs. The term "recruited" implicitly implies that some aspect of the *C. elegans* nervous system is sensitive to the time intervals (the ISIs) used during training and that this process then either directly or indirectly recruits other cellular processes ultimately underlying the development of habituation. There are a number of oscillators (e.g., defecation and egg-laying) in the *C.*

elegans nervous system that could serve such a purpose. According to the hypothesis of Rankin and Broster (1992) the neurons of the TWR circuit are sensitive to the ISI used during training and encode that information. No known oscillators in *C. elegans* are specifically localized to the sensory neurons. This of course does not rule out the possibility that they do in fact exist nor does it mean that they would have to be localized on the sensory neuron. A more parsimonious explanation, one that does not specifically require sensitivity to ISI, may be that several cellular processes are recruited at more than one ISI. Their effects on behavior on the other hand may only be observable with particular training parameters. To illustrate, the effects of the absence of *avr-14* on sensory neuron excitability with repeated stimulation may be occurring at both short and long ISIs. The difference may be that at long ISIs the cell has returned to baseline levels by the time the next tap occurs while at a short ISI it may still be in a state of decreased excitability. Perhaps that is why there are more rapid response decrements with repeated stimulation at short but not at long ISIs. This speculation is in no way intended to argue against the validity of the hypothesis of multiple short-term memory systems. On the contrary it agrees with the idea that certain processes within the nervous system will play a dominant role in habituation to certain training parameters but not others. However, rather than assuming that these are specifically recruited it is suggested that at different ISIs different processes dominate the activity of the neurons underlying the behavior and thus determine the behaviourally observable outcome. This explanation bares some resemblance to the Multiple-Time-Scale (MTS) Model of habituation (Staddon & Higga, 1999). The MTS model's basic premise is that every stimulus creates a unique neural trace and the strength of traces dissipate with time. With short time intervals between stimuli the trace created by the previous stimulus is still active when the subsequent stimulus is presented, resulting in diminished reflex strength, or responding, to the second stimulus. On the other

hand with increasingly longer time intervals between stimuli the trace created by the previous stimulus is increasingly likely to have dissipated when the next stimulus is presented, resulting in higher levels of responding to the second stimulus. The MTS model would predict that increasing the neural trace of the first stimulus in a series of stimuli should result in even less responding when the next stimulus is presented, as the larger trace is more likely to be stronger and have dissipated to a lesser extend at that time. The consequence of this would be decreased responding, and increased rates of habituation across repeated presentation of stimuli. *avr-14* appears to be involved in the regulation of glutamate transmission at the sensory neuron interneuron synapse of the TWR circuit. It was hypothesized that the absence of *avr-14* on the sensory neuron results in increased excitability of the cell and graded increases in glutamate release. This can be thought of as an increase in the strength of the trace that each tap stimulus creates. As these traces are then stronger when the subsequent stimulus is presented, responding to that stimulus is decreased. This may result in more rapid rates of habituation in *avr-14*.

From a functional perspective it seems reasonable for an organism to have multiple short-term memory systems. In habituation to an insignificant stimulus it would be more adaptive to habituate rapidly to the same stimulus presented at a very high frequency than at a lower frequency. Without habituation the repeated energy expenditure caused by full reactions to the stimulus every time within a short time period would quickly accumulate to exhaust the system. Therefore, having a system that is specifically devoted to decreasing reactivity under these circumstances seems adaptive. At long ISIs maintaining full response levels is not as costly to the organism which may be reflected in the fact that habituation occurs at a slower rate at long ISIs. Perhaps a system or process that only comes into play under these circumstances may be equally adaptive.

The results from Experiments 2,3,4, and 5 illustrate the role *avr-14* plays in long-term memory for massed and distributed habituation training. No firm conclusions can be drawn on the role of *avr-14* in 24-hour memory for massed habituation training. Perhaps this should not come as a surprise, as wild-type worms do not show evidence of retention for massed training at 24 hours either. As suggested, differences may be observable at 12 hours, a time period at which wild-type worms do in fact show significant evidence of retention for massed training (Rose and Rankin, 2002a). It appears that the main difference with regards to long-term memory for distributed training between wild-type worms and *avr-14* mutants is that the absence of *avr-14* from the TWR circuit allows for a form of long-term memory not usually seen in wild-type worms. Whether this should in fact be viewed as a unique form of long-term memory is unclear at this point. It appears that there are similarities between memory for distributed training at a 10s ISI in *avr-14* and distributed training at a 60s ISI in both wild-type and *avr-14*. In these experiments the shared aspect of protein synthesis dependence was clearly demonstrated. This implies, though by no means proves, that the same postsynaptic mechanisms are involved in both situations. The suggested experiments on the importance of postsynaptic glutamate receptors will prove crucial for further investigation of this possibility. Assuming that the same postsynaptic mechanisms are in fact recruited by the 10s ISI distributed training protocol, then by elimination the differences that are allowing for the formation of long-term memory with this protocol must be presynaptic. The result of this presynaptic change appears to be the removal of a process whose presence in wild-type worms blocks a transition to long-term memory. What this process is cannot be answered based on the current series of experiments but it seems very likely that glutamate transmission is being affected. Similar to short-term habituation, with repeated 10s ISI stimulation during training blocks in *avr-14* worms larger increases in glutamate release may be occurring compared to

wild-type worms under the same conditions. Ultimately, these increases may be initiating the same postsynaptic changes leading to the formation of long-term memory. The current hypothesis assumes a temporally limited effect of *avr-14* absence and/or activation on glutamate release. A clearer understanding of the precise effects of *avr-14* mutations on long-term memory for distributed training with a 60s ISI is crucial. Experiment 1 showed the effects of *avr-14* appear to be temporally limited in the short-term realm and results from Experiment 2 suggest a similar conclusion in the long-term realm. However, the unexpected result obtained in Experiment 3 questions whether the role of *avr-14* in long-term memory is in fact temporally limited. The observed larger response decrements in sham-shocked *avr-14* worms compared to sham-shocked N2 worms should not be ignored. Certainly, repetition of Experiment 2 will shed some light on this issue. Currently, it appears the data suggest that the presence of *avr-14* blocks the formation of long-term memory for distributed training with a 10s ISI and slow down the development of short-term habituation at a 10s ISI. However, if there is in fact enhanced retention in *avr-14* for training with 60s ISIs, then this implies that the presence of *avr-14* compromises the formation of long-term memory for training with 60s ISIs as well. Absence of *avr-14* should then lead to enhanced long-term memory. The presence of *avr-14* on the other hand may interfere with the formation of long-term memory, but ultimately the presence of other process leads to its formation.

Understanding the processes that *avr-14* is involved in should be an important future endeavour of high priority. The suggestions made with regard to the ISI-dependent effects of *avr-14* mutations are testable. Recent technological advances have made it possible to record from *C. elegans* neurons (Avery, Raizen, & Lockery, 1995; Goodman et al., 2002; Goodman, Hall, Avery, & Lockery, 1998; Lockery & Goodman, 1998). This technique can be used to directly test some of the predictions made. The first step would be to record activity in the

interneurons of the TWR circuit with repeated tap presentation. This would allow for the precise quantification of changes occurring in interneurons as a result of habituation. Further, in the same manner, two specific aspects of neuronal physiology need to be investigated in *avr-14* mutants. First, are there in fact, as suggested, higher levels of depolarization with repeated presentation in the sensory neurons of *avr-14* mutants compared to wild-type worms? Second, does this heightened excitability of sensory neurons dissipate over the course of sixty seconds? Third, how does repeated depolarization of sensory neurons in *avr-14* mutants affect the interneurons at short and long ISIs? At this point there is clear evidence for an ISI-dependent effect of *avr-14* mutation on short-term memory, but obtaining answers to the above questions may add a physiological basis for this ISI-dependence.

Obtaining answers to the questions posed on the role of *avr-14* in long-term memory, particularly for the 10s ISI distributed training protocol, can also be realistically answered. Another approach that could be taken, in addition to the already suggested pharmacological blockade of postsynaptic *glr-1* receptors during training and 1-hour rest periods, would be to test long-term memory for distributed training with a 10s ISI in a *avr14; glr-1* double mutant. Formation of long-term memory is expected to be blocked in such a genetic background. These results are expected to complement those obtained in the experiment involving pharmacological blockade of *glr-1* receptors. Moreover, use of a AVR-14;GLR-1:GFP transgenic mutant would allow for the quantification of postsynaptic changes resulting from distributed training with a 10s ISI, and assessing whether these are in fact the same as those seen with other training protocols.

Even though *avr-14* and other glutamate-gated chloride channels are unique to invertebrates (Cully et al., 1996) there exist structurally similar receptors in vertebrates (i.e., GABA, glycine, and nicotinic acetylcholine receptors). These may also share functional

similarities with glutamate-gated chloride channels in *C. elegans* and other invertebrates. Further, there may exist similarities in the types of processes that glutamate-gated chloride channels in *C. elegans* and similar types of receptors in other species are involved in. Establishing what these processes are is, especially given the technology available now, considerably easier in the simple nervous system of *C. elegans* than the vastly more complicated vertebrate nervous system. If the role of these processes in learning and memory can clearly be understood using *C. elegans* as a model system then this suggests a role for similar processes in learning and memory in more complex systems. Ideally what is required is a comparably well understood vertebrate neural system known to underlie a type of learning and memory. An example that comes to mind is the acoustic startle reflex in rats. On a behavioural level habituation in this system has been well documented (Davis, 1970). Though the neural basis of habituation of the acoustic startle reflex in rats is not understood, the neural circuitry mediating the response is understood quite well (Koch & Schnitzler, 1997). Among vertebrate systems mice have proven themselves as the organisms of choice for studying the effects of gene mutations on behavior. The behavioural parameters of habituation in mice very likely follow those seen in rats and other organisms and there may also be similarities in the neural circuitry underlying the startle response. Mice may thus lend themselves well to investigations of gene mutations on habituation of the acoustic startle reflex. If receptors or associated processes with similarities to *avr-14*, or any other type of receptor or process implicated in habituation in *C. elegans,* exist in components of this circuit then manipulating them either genetically or pharmacologically may lead to similar effects on behavior.

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