SHORT-TERM MEMORY FOR TAP-HABITUATION IN
THE ROUNDWORM C. ELEGANS:
RESPONSE-COMPONENTS & GENETIC DISSECTION

by

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Abstract

Habituation is a simple form of learning that manifests as a decrease in an innate response to a repeated stimulus that is not associated with important events. In this thesis, habituation-memory was studied using the tap-reversal response emitted by the roundworm *C. elegans* in response to a non-localized tap-stimulus. Delivery of 30 tap-stimuli produced a decline in the probability, duration, and speed of the reversal-response (i.e., learning). When stimulation was ceased for 10-minutes each of these response-components recovered partially but not completely, as evidenced by differences between training and test sessions (i.e., memory). Each of the components (probability, duration and speed) of the tap-reversal response showed different profiles of habituation-learning and habituation-memory in wild-type worms, with one not being predictive of the other. A genetic screen supported these findings and identified mutant strains that were deficient in habituation-memory for one component of the response but not the others. It was also shown that simply because a mutant strain showed more habituation-learning did not necessarily predict that it would show more habituation-memory. This suggested that different biological underpinnings likely underlie A) the persistence of habituation from stimulus-to-stimulus within a session of stimuli and B) the persistence of habituation from one session of stimuli to another. The genetic screen also identified mutant strains which supported a genetic dissociation of initial response-difference from overall average response-difference. This suggested that considering the phase of test-session is important for measuring memory. Finally, it was shown that the habituation-memory deficits shown by one mutant strain (*pde-4*) were selective to the frequency of training used and did not show deficits at all training frequencies. Together, these data suggest that habituation-memory is multidimensional and that considering both the components and time-scales of the response is important.
Lay Summary

Learning to repress an innate response to a repeated stimulus is called habituation. This thesis studied the locomotor “reversal-response” that a microscopic worm emitted to a tap-stimulus. When the tap-stimulus was repeated, the response declined. If stimulation was stopped for 10 minutes, then the response recovered partially but not completely, indicating habituation-memory. The habituation-memory was parsed into different dimensions – i.e., probability, duration, and speed of reversal-response. Each of the dimensions showed different rates of habituation-learning and different ways that habituation-memory could be expressed. Phenotyping mutant strains of worms suggested that different genes underlay these dimension-specific forms of habituation-memory. Moreover, the extent of habituation-learning measured within a response-dimension did not predict the extent of habituation-memory. Another finding was that the habituation-memory deficits shown by mutant worm strains could be specific to the temporal parameters of training. Together, these data suggest that habituation-memory is not unidimensional, neither within a single response nor across different time-scales.
Preface

This thesis is based on work conducted in Catharine Rankin’s lab in the Djavad Mowafaghian Centre for Brain Health, Koerner Pavilion at UBC hospital. I was responsible for the conception and design of the experiments, as well as the acquisition and analysis of the data. Some of the data included were collected by undergraduate research assistants (Fung C. and Wong J.), under my supervision, who were members of my team.
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List of Abbreviations

AC = Adenylate/adenylyl cyclase
AMPAR = $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA = Analysis of Variance
ATP = Adenosine triphosphate
BOLD = Blood oxygen dependent level dependent
C. elegans = Caenorhabditis elegans
Ca$^{2+}$ = Calcium
CAMK = Calcium/calmodulin-dependent protein kinase
cAMP = $3', 5'$-cyclic adenosine monophosphate
CBP = (CREB-binding protein)
CDA = Contralateral delay activity
CLASP = cytoplasmic linker associated protein
CREB = cAMP response element-binding protein
CS = Conditioned stimulus
DS = difference-score between average training and test responses (normalized to initial level)
DYRK1A = dual specificity tyrosine phosphorylation regulated kinase 1A
EEG = Electroencephalography
EPSP = Excitatory postsynaptic potential
ERK = Extracellular signal–regulated kinase
fMRI = Functional magnetic resonance imaging
GABA = Gamma-aminobutyric acid
GOF = gain of function
HFS = High-frequency stimulation
HSR = Head shake response
ISI = Interstimulus Interval
LFS = Low-frequency stimulation
LOF = loss of function
LTD = Long-term depression
LTP = Long-term potentiation
MAPK = Mitogen-activated protein kinase
min = minutes
MMP = Matrix metalloproteinases
MMP = Million Mutation Project
MnbK = minibrain kinase
MWT = Multi-Worm Tracker
N = sample size
NGM = Nematode growth medium
NMDAR = N-methyl-D-aspartate receptor
NS = not significant
PFC = Prefrontal cortex
PKA = Protein kinase A
SAPK = Stress-activated protein kinase
sec = seconds
Tukey HSD = Tukey honest significant difference
US = Unconditioned stimulus
Glossary

Between-session habituation = Habituation that persists beyond the training-session and is evident in a test-session, as measured by the arithmetic-difference between the average response emitted during training and test sessions (a measure of memory).

Potentiation of habituation = Habituation that is more rapid and/or more pronounced after multiple series of stimulus-repetitions and spontaneous recoveries.

Spontaneous recovery = the extent to which the initial response assessed post-habituation after a delay is similar in amplitude to that of the original naïve response.

Within-session habituation = Habituation that develops within a single training-session of stimuli, as measured by the arithmetic-difference between initial response and final response (a measure of learning).
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To those that believe that the world can be understood and so try to understand it.
Chapter 1: Introduction

"The simplest, most universal form of learning is habituation. It may be as fundamental a characteristic of life as DNA.”
(Dethier, 1976, p. 411)

"…nobody cares much about sensitization or habituation anyhow."
(McConnell, 2013, p. 311)

1.1 Habituation is a Simple Form of Learning/Memory

Habituation is a form of non-associative learning that appears as a gradual decrease in one or more components of an innate response to a repeated or prolonged stimulus. In lay terms, it has been conceived of as learning to “ignore”, or “repress” a response to an irrelevant stimulus that is not associated with other stimuli/events that have obvious biological consequences. Habituation, like DNA, is found ubiquitously across every phylogenetic level, ranging from protozoa to multicellular eukaryotes (Thompson 2009; Gagliano et al., 2014). Moreover, even within a single organism, many responses—both small (e.g., heart-beat, skin conductance) and large (e.g., patellar reflex, full-body startle)—habituate with repeated stimulation (Hollis, 1971; Meincke et al., 2004; Schoen et al., 2008). The broad ubiquity of habituation both across phylogeny and across levels of behavior is remarkable and is clearly what spurred Dethier’s comparison of habituation to DNA (Dethier, 1976). Despite this, habituation has not enjoyed the same focus within research—as made clear by McConnell’s description of the apathy held towards habituation by many researchers (“…nobody cares…much about habituation”: McConnell, 2013, p. 311). That the ubiquity of habituation is not matched by its study is attributed to at least three reasons: 1) traditional cognitive learning-theory defines memory in terms of reproduction of propositional content (Anderson, 1996), a notion that does not easily map onto habituation phenomena; 2) even to behaviorists working under broader definitions of memory, habituation is
odd because it manifests as a decrease in a pre-existing (innate) response whereas most forms of learning manifest as an increase in a novel response (Rescorla & Holland, 1979; Figueredo, Hammond, & McKiernan, 2006); 3) response-declines are not unique to habituation since systematic decline can also be produced by factors unrelated to memory, such as sensory adaptation (obstruction of stimulus detection), motor fatigue, or damage. In contrast, other forms of learning and memory – both complex (e.g., Pavlovian conditioning) and simple (e.g., sensitization) – produce behavioral changes that are less susceptible to confound by non-learning factors (Rescorla & Holland, 1979).

Despite these conceptual and technical issues, the study of habituation has contributed much to understanding the ecological function as well as neurobiological underpinnings of behavioral plasticity. For instance, within naturalistic settings habituation has been shown to optimize predator detection as well as facilitate mate procurement (Deecke, Slater, & Ford, 2002; Peeke & Figler, 1997; Dong & Clayton, 2009; Hemmi & Merkle, 2009). These studies are complemented by laboratory studies that have characterized the genetic and circuit processes underlying habituation, many of which are shared with other forms of learning/memory. Habituation is also relevant because it is often reported to be altered in neuropsychiatric disorders, with social dysfunction sometimes correlating with habituation impairment (McDiarmid, Bernardos, & Rankin, 2017). As such, there are numerous reasons – both theoretical and pragmatic – for investigating habituation. As a simple model of learning/memory, the study of habituation should facilitate the identification of concrete and systematic principles which may generalize to other forms of behavioural plasticity. Before discussing the biology underlying habituation, a review of its behavioural characteristics is provided.
1.2 Behavioural Characteristics of Habituation

Habituation phenomena show an array of behavioural characteristics that appear in most response-systems (Table 1: adapted from Rankin et al., 2009; Thompson & Spencer, 1966):

Table 1: Behavioural Characteristics of Habituation

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<td>2.</td>
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<td>3.</td>
<td>Potentiation of Habituation</td>
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<td>4.</td>
<td>Sensitivity to Rate of Stimulation</td>
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<td>5.</td>
<td>Stimulus Intensity</td>
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<td>6.</td>
<td>Below Zero Effects</td>
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<td>7.</td>
<td>Stimulus-Specificity</td>
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<tr>
<td>8.</td>
<td>Dishabituation</td>
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<td>9.</td>
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Of these characteristics, three are used to discriminate habituation-learning as responsible for the response-decline as opposed to sensory adaptation, motor fatigue, or damage. These diagnostic criteria are 1) Recovery by Dishabituation; 2) Sensitivity of Spontaneous Recovery to Rate of Stimulation; 3) Stimulus-specificity. Dishabituation occurs when presentation of a novel stimulus (after the response has declined) increases/recovers the response to the original stimulus: i.e., more so than would occur “spontaneously” if recovery proceeded by time alone. In other words, dishabituation provides proof-of-principle that the response is intact and executable but reduced or repressed by some intrinsic process. Response-declines produced by sensory adaptation or motor fatigue do not recover more quickly in response to novel stimulation.

The acquisition of habituation and its rate of spontaneous recovery are also sensitive to the rate of stimulation (i.e., the interstimulus-interval [ISI]) delivered within a training session (Broster & Rankin, 1992). More frequent stimulation produces more response-decline, and more rapid spontaneous recovery. In other words, spontaneous recovery is inversely correlated with the amount of within-session response-decline. This is the exact opposite of what would be expected if sensory adaption or motor fatigue were the cause of the response-decline. One final feature of a response-decline that characterizes it as habituation is stimulus-specificity. Stimulus-specificity is shown by observing that habituation to one stimulus does not alter the response to a different stimulus. In other words, the response-decline is not general but occurs only to the original stimulus that was repeated. If the response-decline were due to a non-learning factor (e.g., motor fatigue) then it would be expected to persist if the identity of the stimulus were changed. Therefore, if a response-decline shows 1) dishabituation, 2) spontaneous recovery that is inversely correlated with the extent of decline, and/or 3) stimulus-specificity, then habituation
is strongly suggested. Alternative accounts, including sensory adaptation (i.e., obstruction of stimulus detection), motor fatigue, or damage are ruled less likely to be primarily responsible.

One feature of habituation that is relevant to this thesis is Potentiation-of-Habituation. This describes the observation that with multiple series of stimulation and spontaneous recovery the rate of response-decline is faster and/or more pronounced (Rankin et al., 2009). Intriguingly, potentiation can be observed even if the response has spontaneously increased (“recovered”) with time to the original baseline level (e.g., Wyers, Peeke, & Herz, 1973; Cheevers & Koshland, 1992). This indicates that spontaneous recovery to original baseline levels does not necessarily mean that the response-system has reset to a naïve state: i.e., it has not truly recovered. Indeed, faster re-learning is a well-established measure of learning/memory and was first described as “savings” by Ebbinghaus (1885) who observed that the rate of learning nonsense syllables increased with experience. Considering this finding, it may be problematic and/or misleading to describe an increase to baseline levels as spontaneous “recovery.” However, because this is the standard use of the term in the field of habituation, I will continue to use it in this way with the caveat that recovery to baseline does not necessarily entail that the system has returned to a naïve state. Unless specified otherwise, spontaneous recovery will be used to describe the extent to which the initial response (test) assessed after habituation is similar in amplitude to that of the original naïve response.

1.3 Biological Mechanisms Underlying Habituation

Two obvious candidates for a cellular mechanism of habituation are depression-of-excitation and potentiation-of-inhibition of the neural pathways that connect sensation to response. Research within the last 50 years – especially in invertebrate species such as Aplysia, Drosophila, and C. elegans – has largely supported the first mechanism (Kupfermann, Castellucci, Pinsker, &
Kandel, 1970; Giles & Rankin, 2009). Depression of excitation occurs intrinsically (homosynaptically) in the shortest pathway connecting sensory neurons to response. It has been hypothesized that habituation is mediated by the inhibition of neurotransmitter-release induced by the inactivation of pre-synaptic voltage-gated Ca\(^{2+}\) channels (Klein & Kandel, 1980; Forsythe, Tsujimoto, Barnes-Davies, Cuttle, and Takahashi (1998); however, additional studies suggested that changes in calcium influx may be insufficient to account for all forms of habituation (Gingrich & Byrne, 1985; Armitage & Siegelbaum, 1998). Some habituation studies have also supported a role for potentiation of inhibition (e.g., Krasne & Teshiba, 1995; Goldberg & Lukowiak, 1984; Stopfer & Carew, 1996; Das et al., 2011). In this scenario, the excitatory signal between sensory neurons and response is suppressed extrinsically (heterosynaptically) by an inhibitory signal. Candidates for this inhibitory signal include GABA (Das et al., 2011; Krasne & Teshiba, 1995) as well as neuropeptides (for review, see Goldberg & Lukowiak, 1984).

### 1.4 Measuring Short-Term Habituation-Memory

Habituation-memory can be expressed in many ways, including spontaneous recovery to below baseline (i.e., initial response-difference: Figure 1: A, B), faster rate of re-habituation (Figure 1: C, D), and/or changes in the final asymptotic level observed within the stimulation session (Figure 1: B, D). These different possibilities are not exhaustive but may be combined. For instance, recovery below baseline (Figure 1: A, B) may be accompanied by faster re-habituation (Figure 1: C, D). It is important to recognize that “potentiation of habituation” has been used inconsistently in the literature to variously describe both faster re-habituation and/or consistently smaller responses.
Figure 1: Different Ways in which Habituation-Memory May be Expressed. A) Memory is expressed as spontaneous recovery to below baseline followed by naïve-like responses. B) Memory is expressed as spontaneous recovery to below baseline followed by consistently smaller responses. C) Memory is expressed as spontaneous recovery to baseline followed by faster re-habituation to the asymptotic level D) Memory is expressed as spontaneous recovery to baseline, followed by faster re-habituation to a lower asymptotic level.
To measure these different forms of habituation-memory, various operational definitions have historically been used: 1) Differences between initial response emitted during training and test sessions; 2) Overall response-differences between training and test sessions. 3) Differences between the slope of response-decline of training and test sessions; 4) Differences in the number of stimuli to reach asymptote.

Instances of faster re-habituation despite recovery to baseline suggests that different mechanisms may underlie each of them (Cheevers & Koshland, 1992; Wyers et al. 1973). Wyers et al. (1973) interpreted recovery to below baseline as a “short-term effect” in contrast to faster re-habituation as evidence of long-term “true retention” so long as it is concomitant with recovery to baseline. However, there is no definitive evidence to support this ranking system of measures since there are examples of habituation in a variety of organisms where spontaneous recovery to below baseline persists for periods as long as 24-hours (e.g., Wright et al., 2009; Rose et al., 2002). Likewise, there are instances of faster re-habituation (despite recovery to baseline) being observed at intervals as short as 2-min (Corfas & Dudai 1989). Number of stimuli to reach asymptote measures both the development and persistence of habituation in terms of the number of stimuli required to reach asymptote.

Each the four measures offers different strengths for assessing memory. Initial response-differences may detect acute memory effects which are local but not distributed across the stimulation session (i.e., if the only difference between training and test sessions were found in the initial response). In contrast, overall-response differences may capture subtle memory effects which are only detectable when aggregating all of responses elicited within a stimulation-session. Similarly, there are different benefits offered by measuring rate of re-habituation with slope-analysis compared to number-of-stimuli-to-reach-asymptote. Assessing for steeper slopes...
is simpler and more generalizable to different paradigms. However, it may mask changes in the asymptote which may be theoretically interesting (Figure 1D). Slope-analysis also does not easily accommodate comparison among groups with different initial response-levels but similar asymptotic levels: i.e., because one group is already closer to the shared asymptote, there will necessarily be a smaller range within which it may show faster re-habitation. In contrast, number-of-stimuli-to-reach-asymptote controls for some (but not all) group differences in within-session habituation. However, it also may mask possible changes in asymptote (Figure 1D).

1.5 Short-Term Habituation-Memory

Short-term habituation-memory has been demonstrated in various systems, including spiders, crabs, fruit flies, sea slugs, nematodes, rats, praying mantids, spiders, isolated sensorimotor synapses, and neuronally differentiated cell lines. The diverse range of response-systems used to study short-term habituation-memory is matched by the variety of sensory modalities involved. For instance, visual stimuli have been used to elicit habituating locomotor defensive responses in both crabs, fruit flies, and praying mantids (Tomsic, & Maldonado, 1990; Tanouye & Wyman, 1980; Balderrama & Maldonado, 1971). Tactile stimulation (e.g., air stream or gentle body touch) has also been used in habituation-memory paradigms in rats (“head-shake-response”), sea slugs (“gill-withdrawal reflex”), nematode worms (“tap-reversal response”), as well as fruit flies (“cleaning reflex”) [Askew, Leibrecht, & Ratner, 1969; Kupfermann et al., 1970; Rankin, Beck, & Chiba, 1990; Vandervorst & Ghysen, 1980)]. Repeated delivery of auditory stimuli has also been used to study memory for habituation of an investigatory response in spiders (Peckham & Peckham, 1887). Perhaps the simplest system in which short-term habituation-memory has been studied is the neuronally differentiated P12 cell line. In this paradigm, repeated stimulation of
single neurons with puffs of ATP, ACh, or electrical pulses causes habituation of norepinephrine-release (Cheevers & Koshland, 1992). Habituation-memory in this model was studied as faster re-habituation following recovery to baseline. Interestingly, it was shown that faster re-habituation required different amounts of stimulation depending on whether ATP or ACh were used.

In *Drosophila*, two notable genes have been identified that affect short-term habituation-memory. These genes are *rutabaga* (adenylyl cyclase) and *dunce* (phosphodiesterase) which respectively synthesize and degrade cAMP levels. In the escape response paradigm, a light-off stimulus triggers leg extension and flight initiation which habituates with repeated stimulation. With a retention interval of 5-sec between stimulus-blocks, recovery to baseline was observed in controls and *rutabaga* but not in *dunce* mutants (Engel & Wu, 1996). However, despite recovery to baseline, controls, *rutabaga*, and perhaps *dunce* showed faster re-habituation. At a longer retention interval of 2-min, no evidence of memory was found in control or *dunce* mutants, however, *rutabaga* mutants continued to show faster re-habituation.

Evidence that *rutabaga* may affect retention of habituation – specifically in the augmented rate – is found in a different *Drosophila* paradigm, called the “cleaning reflex response.” In this paradigm, tactile stimulation of thoracic bristles elicits a sweeping leg movement that resembles cleaning. With repeated stimulation, this cleaning reflex shows habituation and is retained (across a delay) as recovery to below baseline and faster re-habituation (Corfas & Dudai 1989). Faster re-habituation in *rutabaga* mutants decayed much faster than controls, showing naïve-levels as early as 5-min. While these results could be interpreted as indicating that *rutabaga* and *dunce* show enhanced memory under certain circumstances, it is important to stress that these mutations also altered the acquisition of
habituation. Indeed, *rutabaga* required ~10-fold the number of stimuli to reach asymptote criterion whereas *dunce* required less than half (Engel & Wu, 1996). As such, *rutabaga* (adenylyl cyclase) and *dunce* (phosphodiesterase) may be considered learning and/or memory mutants, depending on the paradigm.

The rodent “head-shake-response” (HSR) is another notable paradigm of habituation that has been used to investigate short-term memory and its underlying biology. Rats receive a stream of air to the ear to which they rotate their head. When air-streams are repeatedly delivered, this response declines and shows habituation-memory beyond the initial stimulation-session, as evidenced with a second stimulation-session. Memory for HSR-habituation is expessed at 5-min and 2-hr intervals as spontaneous recovery to below baseline and/or faster re-habituation (Murphy, Harding, Muhunthan, Holtfreter & Wright, 2005; Wright et al., 2006). These behavioural markers of memory were accompanied by brain-area-specific increases in expression levels of several mitogen-activated protein kinases (MAPKs) and matrix-metalloproteinases (MMPs). With respect to MAPKs, robust increases in p-38 and SAPK levels were found in the hippocampus, cerebellum, prefrontal cortex, and piriform cortex at 5-min and 2-hrs after habituation-training (Murphy et al., 2005). The other MAPK assessed, ERK, only showed moderate elevations that were only observed at 5-min after training. With respect to matrix-metalloproteinases, MMP-3 and MMP-9 showed habituation-induced increases within the hippocampus 2-hr but not 5-min after training (Wright et al., 2006).

These correlative data were made more compelling by demonstration that delivery of MMP-3 inhibitors facilitated spontaneous recovery (Wright et al., 2009). The role of the hippocampus in subserving HSR habituation-memory is also supported by the suggestion that hippocampectomy may facilitate spontaneous recovery without altering habituation itself.
(Wright et al., 2004). While persistent habituation of the HSR is produced with a single ~10-min training-session, it is interesting that changes in gene expression (i.e., MAPKs) are measured as early as 5-min post-training. Short-term memory in other animal and cellular models usually does not require protein synthesis (though see Schilhab & Christoffersen [1996] who detected protein-synthesis dependent synaptic depression in Helix pomatia only 6-10 min after a series of EPSPs). Therefore, whether habituation-memory for the HSR involves the same mechanisms as in other short-term memory paradigms in animals is open to debate. Nonetheless, these studies in both Drosophila and in rats demonstrate that short-lasting forms of habituation may involve distinct genetic underpinnings.

1.6 Other Paradigms for Studying Mechanisms of Short-Term Memory

In addition to habituation there are many other paradigms for studying short-term memory – at both behavioral and cellular levels – that have contributed much to identifying the underlying mechanisms. Within behavioral neuroscience, paradigms of short-term memory have often made use of non-declarative tasks since they are amenable to experimentation in animals: i.e., because explicit recollection is not required, only motor performance. For instance, short-term forms of classical and operant conditioning exist as do short-term forms of sensitization (all examples of non-declarative memory). These behavioral paradigms of short-term memory are complemented by cellular paradigms that identify stimulation-dependent changes in synaptic efficacy (post-tetanic potentiation, synaptic augmentation, short-term facilitation, early-phase long-term potentiation) between neurons in culture or tissue slices.

Together, these behavioural and cellular paradigms of memory have yielded two key insights that appear to delineate short-term forms from long-term forms in all systems investigated: Short-term memory develops within a single training-session and involves covalent
changes in the activity of ion-channels, receptors, and second-messengers related to calcium (Dudai 2004). In contrast, long-term memory usually involves spaced-training and requires protein-synthesis dependent structural changes in neurons, through changes in the number and location of receptors, ion channels, and synapses (Dudai 2004).

Aversive olfactory conditioning in *Drosophila* is one example that demonstrates the mechanistic differences underlying short- and long-term memory. In this memory-task, flies are exposed to two odours, one of which is associated with an electric shock. After multiple trials of this selective odour-pairing, flies learn to selectively avoid the one paired with shock. The canonical underlying model suggests that within the mushroom body adenylyl cyclases (ACs) integrate Ca\textsuperscript{2+} evoked by the conditioned-stimulus (CS) with G-protein-coupled activation evoked by the unconditioned-stimulus (US) [Waddell & Quinn, 2001]. Together, the CS and US synergistically increase the amount of cAMP that is produced by ACs (Tomchik & Davis 2009). Elevated cAMP levels, in turn, activate protein kinase A (PKA) which subsequently is thought to phosphorylate ion channels (e.g., Na\textsuperscript{+} and K\textsuperscript{+}) thereby altering neuron excitability. In contrast, long-term forms of aversive olfactory conditioning in *Drosophila* require protein synthesis and can be prevented by heat shock or cycloheximide (Tully, Preat, Boynton, & Del Vecchio, 1994).

Perhaps the most well-established cellular model of short-term memory has relied on isolated sensorimotor synapses of *Aplysia*. In a simple version of this preparation, a sensory neuron, a motor neuron, and an intervening serotonergic “facilitator” neuron are co-cultured together *in vitro* (Rayport & Schacher, 1986). Current is injected into the sensory neuron and the post-synaptic potential is measured on the receiving end within the motor neuron. Low-intensity stimulation of the sensory neuron produces short-term synaptic depression, the cellular analogue of short-term habituation. In contrast, stimulation of the serotonergic facilitator neuron or direct
bath application of serotonin produces short-term synaptic facilitation, paralleling short-term sensitization in vivo (Rayport & Schacher, 1986). With this cellular preparation, many of the critical molecular processes first identified in Aplysia and in Drosophila have been replicated. Indeed, in Aplysia short-term facilitation in vitro appears to be mediated by serotonin-sensitive AC and cAMP-dependent PKA (Hawkins, Kandel & Bailey, 2006). This, in turn, leads to spike broadening via reduced K⁺ current through channel phosphorylation that increases Ca²⁺ influx at the terminals. In contrast to short-term facilitation, it appears that short-term depression involves reduced Ca²⁺ influx during the action potential (Eliot, Hawkins, Kandel & Schacher, 1994).

While these in vitro and in vivo mechanisms of short-term memory continue to guide and inform research in animal models, it is somewhat unclear to what extent they map precisely onto human models of learning. This may be because of differences in the tools available to researchers (i.e., invasive/genetic vs. non-invasive/fMRI) but also because of differences in the memory-tasks investigated. Whereas animal research has been limited primarily to studying non-declarative memory, human research has focused on tasks that demand explicit recollection. For instance, participants are typically asked to recall items (e.g., letters) or detect changes in a discrete stimulus-set (e.g., an array of colours) [e.g., Luck & Vogel, 1997]. This distinction between memory-task is also reflected by the different terminology that is employed by cognitive neuroscience and cellular neuroscience. Within cognitive neuroscience, “working memory” appears to have largely replaced the term short-term memory and has a somewhat more specific meaning (Squire & Dede, 2015). Working memory is often viewed as a capacity-limited holding system of task-specific information that rapidly decays unless it is maintained in attention (Gathercole, 2007). In contrast, short-term memory is sometimes reserved to describe tasks such as immediate serial recall which do not demand much concurrent processing.
As to its biological underpinnings, working memory appears to be related to sustained neural activity that outlasts the physical presence of the stimulus during the delay period (i.e., retention interval). EEG studies show this sustained neural activity comprises 40-hz oscillations that synchronize during the delay period (Miltner, Braun, Witte, & Taub 1999) and are stimulus-specific (Gray, König, Engel, & Singer, 1989) and enhanced by attention (Tiitinen et al. 1993). Moreover, the brain region that is involved depends on the type of the information maintained (for review, see Jonides et al., 2008). For instance, lesions to the temporal cortex appear to impair visual but not spatial working memory whereas lesions to the parietal cortex show the opposite pattern (Owen, Sahakian, Semple, Polkey, & Robbins, 1995; Pisella, Berberovic, & Mattingley, 2004).

However, it appears that the prefrontal cortex (PFC) may be generally responsible for maintaining working memory, especially in the face of distractors. This is supported by both lesion case studies and neuroimaging studies (fMRI and EEG) which demonstrate that working memory is correlated with elevated and sustained activity during the delay period in prefrontal areas. The signals recorded by fMRI and EEG – respectively, blood-oxygen dependent levels (BOLD) and contralateral delay activity (CDA) – also show sensitivity to the number of items in memory. Often the BOLD and CDA responses increase with the load of the memory task, and display sharp discontinuity after a certain item threshold is reached (Linden et al., 2003; Leung, Seelig, & Gore, 2004). This supports the 4 or 7 item capacity limit that is often attributed to working-memory (Cowan, 2000; Miller, 1956). These correlational data are made more compelling by more recent evidence that non-invasive electrical or magnetic stimulation of the
PFC affects working memory in human participants (Brunoni and Vanderhasselt, 2014; Feredoes, Heinen, Weiskopf, Ruff & Driver, 2011).

In sum, there are various paradigms which have been used to investigate short-term memory in both animals and humans. It is unclear currently how to precisely reconcile the different mechanisms that have been identified at the cellular, behavioral (performance), and cognitive level. However, a governing hypothesis made as early as 1894 by Ramon y Cajal is that unifying biological principles underlie all forms of learning/memory. This hypothesis was strengthened by Hawkins & Kandel (1984) who demonstrated that higher-order learning phenomena (e.g., classical conditioning, generalization, second-order conditioning, extinction) could, in principle, be implemented by combinations of the molecular processes that underlie habituation and sensitization in Aplysia. This finding suggested to Hawkins & Kandel (1984) that the mechanisms of yet even higher forms of learning (e.g., insight learning) possessed by a select few organisms may also be elaborations of phylogenetically “older” forms of learning (e.g., habituation) that are conserved across all animals. In support of this, many of the genes identified in invertebrates that play a role in in non-associative learning also play similar roles in associative learning in mammals (Kandel, Dudai, & Mayford, 2014).

1.7 *C. elegans*: Tap-based Habituation

*C. elegans* is a microscopic roundworm with a short-life span of 2-3 weeks and reaches sexual maturity at ~3.5 days. While the natural habitat of *C. elegans* consists of rotting plant matter (Félix & Braendle, 2010), in the laboratory it is reared on agar-filled Petri plates. Normally the worm crawls forward across the surface of the agar; however, if a mechanical tap-stimulus is delivered to the Petri dish, the worm transiently reverses and crawls backwards. This reversal-response declines with repeated tap-stimulation. The response-decline has been shown to
dishabituate (by electric shock) as well as exhibit spontaneous recovery that is inversely related to within-session decline (Rankin, et al., 1990; Rankin & Broster, 1992). Additionally, the response-decline is specific to taps since tap-stimulation does not affect reversals to a heat-stimulus that produces backward movement (Wicks & Rankin, 1997). Therefore, the tap-induced decline in the reversal-response shows all three key diagnostic criteria of habituation that distinguish it from sensory adaptation and motor fatigue. Faster re-habituation of tap-response despite spontaneous recovery to baseline has also been shown (Amano, Kitamura, & Hosono, 1999). The discovery of tap-habituation in a simple organism like *C. elegans* offered a new paradigm through which fundamental learning/memory features – both behavioral and biological – could be uncovered.

### 1.7.1 Behavioural Features of Within-Session Tap-Habituation (Learning)

Tap-habituation most often appears as a negative exponential curve, with two phases (Broster & Rankin, 1992; Kitamura, Amano, & Hosono, 2001): 1) *Descending Phase*: the initial phase of habituation is rapid and the response quickly decreases, 2) *Asymptotic Phase*: in this later phase, the response undergoes a much slower or negligible decrease as it approaches an asymptotic level. Different interstimulus intervals (ISIs) produce different rates of habituation as well as different asymptotic levels. Compared to longer ISIs, shorter ISIs produce more rapid habituation and lower (“deeper”) asymptotic levels (Broster & Rankin, 1992). Importantly, these features of tap-habituation characterize many other forms of habituation in other response-systems and organisms (Rankin et al., 2009).

### 1.7.2 Biological Mechanisms of Within-Session Tap-Habituation (Acquisition)

Genetic screens in *C. elegans* have identified numerous genes that affect response-decline to a series of stimuli (within-session habituation). Of these, many point to homosynaptic synaptic
depression as the underlying mechanism, similar to models of habituation in other organisms. Indeed, most of the genes that affect tap-habituation in C. elegans are expressed directly within the circuit intervening stimulus-detection and response and are known to affect neurotransmission. Some of these genes include eat-4 (glutamate vesicular transporter: Rankin & Wicks, 2000), cat-2 (tyrosine hydroxylase), dop-1 (type 1 dopamine receptor: Sanyal et al., 2004; Kindt et al. 2007), cmk-1 (Ca^{2+}/calmodulin-dependent kinase-1 CaMK1), ogt-1 (O-linked N-acetylglucosamine transferase: Ardiel et al., 2018), and hab-1 (product unidentified: Xu, Sassa, Kunoh, & Hosono, 2002). When mutated these genes alter habituation-kinetics by modifying either the rate or final asymptotic level of response-decline that occurs within a training-session of taps. For example, eat-4, cat-2, and dop-1 worms show an accelerated rate of habituation (as compared to wildtype/N2) whereas hab-1 worms show a much slower rate of habituation. Evidence that the rate of habituation may be genetically dissociated from its asymptotic level was provided by Ardiel et al., (2018). At a 10-sec ISI, cmk-1 and ogt-1 showed a faster rate of habituation but an asymptotic level indistinguishable from wild-type/N2. In contrast, at a 60-sec ISI, these mutants showed a slower rate of habituation with an asymptotic level that was higher than wild-type (Ardiel et al., 2018). The specificity of ISI in observing these genetic effects also supported the hypothesis that different genetic mechanisms underlying habituation are recruited by different ISIs (Rankin & Broster, 1992).

What is also known about the tap-reversal response is that it is mediated by two antagonistic forward and reverse subcircuits that are activated simultaneously by the tap (Wicks & Rankin, 1996a). This was initially suggested by the observation that tap-delivery occasionally evoked a forward crawling response instead of the usual reversal response. Laser ablation assays suggested that the forward crawling response requires input from PLM (posterior)
mechanosensory neurons whereas the reversal crawling response requires input from ALM and AVM (anterior) mechanosensory neurons (Wicks & Rankin, 1996). Comparing habituation-kinetics in these different groups of laser-ablated worms suggested that a) forward and reverse subcircuits habituate at different rates and that b) the intact response (reversal) is the net integration of the two (Wicks & Rankin, 1996). The observation that forward and reverse circuits habituate at different rates suggests that different biological mechanisms may operate within them. Indeed, dop-1 affects habituation by modulating the sensitivity of the anterior ALM mechanosensory neurons (Kindt et al., 2007), by gating the Ca^{2+}-evoked transient evoked by tap. This is important to bear in mind when the cellular locus of a mutation affecting tap-habituation is not precisely identified.

1.7.3 Behavioural Features of Short-Term Memory for Tap-Habituation

Within the tap-habituation paradigm in C. elegans, less attention has been paid to short-term memory that persists beyond the training session. Much of the existing research has operationally defined short-term memory for tap-habituation as the amount of spontaneous recovery in a given period of time. Incomplete recovery to below baseline – i.e., response levels that have not completely returned with time to initial (naïve) levels (Figure 1 A, B) – is interpreted as evidence of memory for the previous training. Parametric behavioural studies have identified a key role of the interstimulus interval (ISI) in determining the persistence of habituation-memory defined as such. By subtracting ISI-specific asymptotic levels from test-responses 30-sec, 10-min, 20-min, and 30-min after habituation training with 60 tap-stimuli, Rankin & Broster (1992) showed that spontaneous recovery could not be accounted for by ISI-specific differences in asymptotic levels; as described earlier, shorter ISIs produce relatively lower (“deeper”) asymptotic levels than longer ISIs. Matching the number of stimuli required to reach asymptote between worms trained
with long and short ISIs also ruled out number of stimuli as being responsible for spontaneous recovery. Once a worm had reached its own asymptotic response level, neither A) the number of tap-stimuli needed to reach asymptote, nor B) the actual degree of response-decline at asymptote could predict the rate of spontaneous recovery. Comparing the rate of spontaneous recovery shown by worms trained at each ISI also suggested that the number of missed stimuli was not a major factor (Rankin & Broster 1992).

As such, tap habituation-memory – as measured by spontaneous recovery to below baseline – was hypothesized to be largely established by achieving the particular asymptotic level that characterized an ISI (Rankin & Broster 1992). Another way of viewing this is that once asymptote is attained, encoding of ISI is “complete” and rate of spontaneous recovery (a measure of memory persistence) may be predicted. It is notable that the ISI used during training influences not only the rate of recovery but also the degree of recovery. Of the parameters assessed by Rankin & Broster (1992), recovery to baseline after 30 min was only observed when extremely short ISIs (i.e., 2-sec) were used during training and when at least 10-min was allowed to elapse after training. At every other ISI assessed (10-sec, 30-sec, 60-sec), only recovery to below baseline was observed (i.e., an increase above asymptotic level), even at the longest recovery-interval tested (30-min). While this suggests the experience of 60 taps persisted for at least 30-min (if given at ISIs as short as 10-sec), more durable forms of tap-habituation lasting as long as 72-hrs have been shown (Rankin et al., 1990; Ebrahimi & Rankin, 2007). However, in line with the focus of this thesis, only short-term forms of habituation-memory are reviewed.

Two other forms of short-term tap habituation-memory include context-modulated habituation memory and re-training memory. These forms of memory last for 1-hour and also rely on spontaneous recovery to below baseline as a measure of retention. Context-modulated
habituation memory describes the observation that the expression of habituation-memory during test is enhanced by presenting the same associative cues that were present during training. At the behavioral level, enhancement manifests as smaller responses to the first two tap-stimuli delivered during test. To produce this effect, worms were trained and tested with 30-taps (at 10-sec or 60-sec ISI) in the presence of a contextual (associative) cue that is olfactory, gustatory, or non-descript (plain agar) [Rankin, 2000; Lau, Timbers, Mahmoud & Rankin, 2013]. Since the effect did not occur if the cue was omitted during testing, it may be appropriate to hypothesize that processes similar to recall are involved, in line with associative models of habituation (Wagner, 1981). The other type of short-term memory for tap-habituation shown in *C. elegans* is called *re-training memory* because it is observed in the training process of inducing *long-term memory* (i.e., tap-habituation that persists for > 24-hrs and which requires protein-synthesis). In this training protocol, worms received blocks of 20 tap-stimuli (at the longer ISI of 60s ISI), separated by 1-hour rest-intervals. The block-average response to tap decreased with each subsequent training-block (Beck & Rankin, 1995; Rose, Kaun, & Rankin, 2002). Hosono and colleagues used a similar method to produce habituation (50 taps at 10-sec ISI) that persisted for 1-hour, as measured by comparing the first 5 test-responses with the initial response (Amano et al., 1999) or number of stimuli to reach asymptote (Xu et al., 2002). These alternative measures reinforce the notion that spontaneous recovery to baseline may not necessarily indicate the absence of habituation-memory. Indeed, with the same training method described above, Xu et al (2002) showed that spontaneous recovery to baseline had occurred by 5-min after stimulation was ceased but was followed by much faster re-habituation.
1.7.4 Biological Mechanisms of Short-Term Memory for Tap Habituation

While less research has focused on short-term memory for tap-habituation in *C. elegans*, even less is known about its biological underpinnings. However, genetic screens of mutants for context-modulated habituation memory and re-training memory suggest a possible role for glutamate signaling. Indeed, *nmr-1* (NMDAR) and *glr-1* (AMPAR) – both receptors for glutamate – are required for context-modulated habituation-memory (Lau et al., 2013). These receptors are expressed in many neurons, including the interneurons of the circuit driving tap-reversals as well as RIM. Less is known about the biological underpinnings of re-training memory; however, it appears that worms with mutations in *glr-1* (AMPAR) and *eat-4* (vesicular-glutamate-transporter) show normal or possibly enhanced re-training memory (Rose et al., 2002).

Habituation in *eat-4* worms is faster and reaches a much lower asymptotic level than wildtype/N2 worms (Rankin & Wicks, 2000). Intriguingly, dishabituation by electric shock does not readily recover the response-decline in *eat-4* worms. While this observation alone might have suggested fatigue/adaptation as being responsible, habituation-processes were confirmed by showing that spontaneous recovery in *eat-4* worms was inversely correlated with ISI-dependent response-decline (Rankin & Wicks, 2000).

Xu et al. (2002) performed the first forward genetic screen for habituation mutants and identified a handful which alter the rate of habituation or its 1-hour persistence. Of about 30,000 mutagenized worms, three mutants were identified and mapped to different genetic loci: *hab-1*, *hab-2*, *hab-3*. Unfortunately, little is known about the molecular function underlying these mutations since these genes were not identified. However, unpublished studies suggest that *hab-1* encodes a subunit of mitochondrial complex I with reduced enzymatic function (e.g., Falk et al., 2005). Xu et al. (2002) reported that (compared to wildtype/N2 worms) *hab-1* worms
habituated slower and showed no 1-hr retention with train and test sessions of 50 stimuli at a 10-sec ISI.

It is important to stress, however, that Xu et al. (2002) used a different operational definition than Rankin et al. to quantify within-session habituation (learning) and between-session habituation (short-term memory). The studies from the Rankin Lab reviewed thus far quantified within-habituation and between-session habituation, respectively, using slope-regression and incomplete spontaneous recovery to below baseline. In contrast, Xu et al., (2002) quantified habituation-memory by counting the number of stimuli required to reach asymptotic level. In this framework, reaching asymptote is conceived as “acquisition of habituation” and memory is evidenced by an increased propensity to reach asymptote. This measure may be problematic given that the initial tap-response emitted by hab-1 worms is much greater in magnitude than N2 worms whereas its final asymptotic level appears to be indistinguishable from N2. Because of their hyperresponsivity, hab-1 worms may simply require more stimuli to reach asymptotic level. Comparing the initial response of hab-1 during training and test curves suggests that these mutants show spontaneous recovery to below baseline, a measure of memory. This may serve as a reminder that our conceptions of memory and its biological underpinnings are strongly dependent on the operational definitions that are chosen. Spontaneous recovery to below baseline and number of stimuli to reach asymptote may represent two types of habituation-memory with distinct genetic underpinnings. It is currently unclear how to compare these measures of memory.

In sum, the genes identified to play a role in short-term memory for tap-habituation in C. elegans include glr-1, nmr-1, hab-1, and, possibly, eat-4. Except for perhaps hab-1 (whose identity is unknown), these genes function in the tap-reversal circuit either in the sensory neurons
(eat-4) or in downstream interneurons (gll-1, nmr-1). The pivotal role that ISI has on spontaneous recovery (reviewed in the previous section) suggests that these genes may alter the recruitment of ISI-dependent processes. Indeed, Wicks & Rankin (1996b) demonstrated that modifying the kinetics of tap-habituation by laser-ablation of the posterior mechanosensory neurons (PLM) did not affect ISI-dependent spontaneous recovery. Whereas animals lacking PLM mechanosensory neurons habituated at a shorter ISI with kinetics that resembled habituation at a longer ISI, they demonstrated spontaneous recovery that was indistinguishable from intact animals trained at a shorter ISI. This suggests that habituation-memory is intimately correlated with the ISI-dependent processes that are recruited during training rather than to the superficial characteristics of the habituation curve (e.g., rate or asymptote). As such, if a biological manipulation such as cell-ablation or genetic-lesion alters spontaneous recovery it may be due to alterations in ISI-dependent processes.

1.8 Aims and Rationale

There are numerous reasons – both theoretical and pragmatic – for investigating short-term habituation-memory. These include ubiquity across phylogenetic and behavioral levels, relevance to neuropsychiatry, and biological relevance to other forms of learning/memory. Because of its simplicity, habituation can be a model of other forms of learning/memory.

My premise is that detailed parametric behavioral analyses can inform and guide the search for biological underpinnings of short-term habituation-memory; if the behavior is ill-defined or simply-defined then our conception of the underlying mechanisms will be correspondingly ill-defined or simple. For many pragmatic reasons behavior is often operationalized using course, quick, and convenient measurements. Because this project investigates a simple, reflex-like response in a small animal with a short-generation time using
high-throughput behavioral tracking, I am able to partially circumvent this issue and rigorously examine its merits.

In Chapter 2, I assess various components of the reversal-response in wild-type/N2 and identified various ways in which 10-minute tap habituation-memory can be expressed. In Chapter 3, I extend this phenotypic characterization and demonstrate that the various forms of tap habituation-memory could be dissected along genetic lines. The profile of tap habituation-memory was found to depend on the following: A) the component that was chosen (i.e., probability, duration, speed), and B) the phase of stimulation that was assessed (i.e., the initial test response following the rest-interval or the overall response during testing). Chapter 4 validates the persistent response-declines observed in each reversal component as genuine forms of habituation and shows that the frequency of stimulation (ISI) used during training plays a role in determining to what extent habituation-memory is observed. The diverse array of ways in which habituation-memory can be expressed suggest that no one single measure can comprehensively represent the phenomenon.

Another aim of this thesis was to determine whether memory (defined with various measures) could be predicted by the overall amount of habituation observed during training. In other words, to what extent did habituation-learning predict habituation-memory? It appeared that, neither within a genotype (Chapter 2) nor across genotypes (Chapter 3), could habituation-memory be easily predicted by the amount of habituation observed during training. Within a genotype, greater within-session habituation in one component over another (e.g., probability vs. speed) did not necessarily predict greater memory in that component. The difficulty in predicting memory from the degree of learning was also reinforced by the identification of mutants which showed altered habituation within the training session but normal memory at test. Conversely,
mutants were identified which showed normal habituation within the training session but abnormal memory at test.

Together these findings suggested that habituation is not unidimensional at different time-scales. Different biological underpinnings likely underlie A) the persistence of habituation from stimulus-to-stimulus within a session of stimuli and B) the persistence of habituation from one session of stimuli to another.
Chapter 2: Parsing Tap Habituation-Memory in *C. elegans* Into Response-Component and Phase of Test-Session

2.1 Introduction

Prior to 2011, research on tap-habituation in *C. elegans* operationally defined the reversal-response as “magnitude” which combined both A) the probability of the reversal-response being elicited by the tap within 1-sec of delivery with B) the distance of the reversal-response into a single measure. With the advent of the Multi-Worm Tracker, an automated machine vision behavioural-tracking device, this aggregated response could be parsed into its various components, such as probability, distance, duration, speed, and latency (Swierczek, Giles, Rankin, Kerr, 2011). In addition to offering enhanced precision, high-throughput tracking also broadens the scope of behaviours that can be studied beyond a focal response since data-collection is no longer a bottleneck. Both of these features – enhanced precision and broadened scope – have contributed to understanding tap-habituation in *C. elegans*.

With respect to precision, Timbers, Giles, Ardiel, Kerr, & Rankin (2013) showed that worm age affected reversal-probability but not reversal-distance. This dissection of magnitude into its components was extended by Giles (2012) who showed that reversal-probability and reversal-duration may also have separate genetic underpinnings. Screening hundreds of mutants carrying putative null alleles for synaptic genes suggested that different genes underlie reversal-probability and reversal-duration. Evidence that response-speed could also be mediated by distinct genes was first provided by Ardiel et al., (2018). Intriguingly, this study also demonstrated that phenotyping mutants at the level of reversal-distance masked underlying differences in duration and speed. Indeed, mutations in the *C. elegans* homolog of O-GlcNAc
transferase (ogt-1) and Ca\textsuperscript{2+}/Calmodulin kinase 1 (cmk-1) showed identical habituation phenotypes when the response was scored as distance; however, closer analysis revealed that while ogt-1 primarily affected duration, cmk-1 primarily affected speed. This genetic-dissection of a reversal-response into probability, duration, and speed was extended even further by Ardiel et al., (2017) who demonstrated using another reversal-response that latency could also be separately modified by genetic lesion. In this paradigm, the reversal-response following by optogenetic stimulation of a pair of nociceptor neurons called ASH was investigated. Repeated stimulation of ASH led to a reversal-response that decreased in duration but which increased in latency. This latter metric of habituation (i.e., increased latency) was severely affected by genetic knockout of a neuropeptide, pigment-dispersing-factor-1 receptor (pdfr-1), which reduced the increase in latency. The Ardiel et al. (2017) study also demonstrates the other feature of high-throughput tracking mentioned earlier – namely, broadened scope of behaviours analyzed. While repeated ASH stimulation produced habituation in the reversal-response it also appeared to sensitize a subsequent forward-response. Critically, this subtle shift in response strategy from reversals to enhanced forward movements was only made apparent with the advent of high-throughput tracking that enabled ongoing behaviour to be continuously measured. In sum, these studies underscore the point that there are numerous ways in which a gene may exert an effect on a behavioural phenotype even for a simple reflex-like response.

Given the phenotypic detail afforded by the MultiWorm-Tracker, I chose to use it to investigate short-term memory for tap-habituation. In the past, short-term memory for tap-habituation has been studied at the aggregate level of response-magnitude (e.g., Rankin & Broster, 1992) or block-averaged response-magnitude (Rose et al., 2002). As reviewed earlier, defining response in terms of magnitude often masks the kinetics of the underlying components
as well as their distinct genetic underpinnings. This issue may be compounded when responses are aggregated into larger blocks of stimuli as with studies showing 1-hr re-training memory [produced at 60s-ISI] (Rose et al., 2002). Indeed, Xu et al., (2002) used a similar paradigm to show that 1-hour re-training memory [produced at 10s-ISI] manifested as recovery to baseline followed by faster re-habituation. This subtle finding would not be identified in habituation studies that define memory using block-responses (e.g., Rose et al., 2002) or define memory using spontaneous recovery to initial level (e.g., Broster & Rankin, 1992).

Together, these data suggest that much is to be gained by A) higher-precision phenotyping and B) memory paradigms that are more extensive than spontaneous recovery. One other rationale that merits investigating short-term memory for tap-habituation is that not much is known about its genetic underpinnings. With these motivating factors, I established a memory paradigm consisting of two sessions of 30-tap stimuli with an intervening rest interval of 10-min. This experimental design was chosen for two reasons: 1) asymptotic phase of habituation (as measured in terms of magnitude) is achieved on average by 30 stimuli (Broster & Rankin, 1992). This is expected to reduce variability since degree/rate of spontaneous recovery (one measure of habituation-memory) is largely determined once asymptote is reached (Broster & Rankin 1992); 2) subjecting worms to two full sessions of stimulation enables any potentiation of tap-habituation to be assessed which provides an additional measure of memory.
2.2 Methods

2.3 Strain Maintenance

Worms were cultured on Petri plates of nematode growth medium (NGM) seeded with 50 uL of *E. coli* (OP50; Brenner, 1974). N2 Bristol *C. elegans* were obtained from the *Caenorhabditis* Genetic Center (University of Minnesota, Minneapolis, MN). Petri plates were filled with agar no more than 2 weeks prior to use. The experiment (age-synchronization, development, and stimulation) was conducted within a room controlled for humidity (~40+/−5 % RH) and temperature (20+/−1 °C). Worms were age-synchronized by transferring gravid worms (5-10) onto plates seeded with 50 uL of *E. coli* the night prior. After 2-4 hours, gravid worms were removed leaving ~40-80 eggs on each plate. In order to minimize extrinsic stimulation before training & testing, these synchronized plates were subsequently wrapped in Parafilm wax and left in Tupperware containers placed on a foam-padded shelf hanging from the ceiling with bungee cords.

2.4 Behavior/Tap-Stimulation

Behavioral experiments were conducted approximately 96 hours (4-days) after eggs were laid when worms were considered adults. The MultiWorm-Tracker was used to automatically deliver taps at an interstimulus-interval (ISI) of 10-sec and collect behavior data. Before tap-stimulation, the Parafilm wax wrapping each plate was removed and the lid of each plate was briefly removed to provide a brief air-puff stimulus to arouse the worms so they could be tracked. 3-10-min after the lifting of the lid, tap-stimuli commenced. Tap-stimulation consisted of a training-phase of 30 stimuli delivered at a 10-sec ISI, a 10-min rest-interval during which no taps were delivered and a test phase of 30 stimuli delivered at a 10-sec ISI: therefore, worms received 60 tap-stimuli in
total. Approximately 90 plates of worms were trained and tested with an average of ~40 worms
tracked per response.

2.5 Operational Criteria for Tap-elicited Reversal-Response and Worm

As in previous research, tap-elicited reversals were operationally defined as reversals that
occurred within 1-sec of the tap being delivered. Choreography software was used to pre-analyze
data collected by the MultiWorm-Tracker. Only objects (worms) existing for a minimum time of
20-sec and moving at least 2 body lengths at some point during the experiment were included in
analysis in order to exclude artifacts. The time-window over which speed was calculated was
0.1-sec. To implement these criteria, the following commands were passed to Choreography: -p
0.027 -s 0.1 -t 20 -M 2 --shadowless -S -o nNss*b12xyMmeSakcr --plugin Reoutline::exp --
plugin Respine --plugin MeasureReversal::tap::dt=1::collect=0.5::postfix=trv --plugin
MeasureReversal::postfix=txt.

2.6 Statistical Analyses

Assessment of a Given Response-Component

The “N” for all analyses was number of plates. This is a more conservative enumeration of
sample size than either i) the number of worms or ii) number of responses collected per plate.
The mean plate-responses to the 60 total stimuli across training and testing were grouped into 30
bins of 2 stimuli each, and the mean of each bin was calculated per plate. These data were then
analyzed using a 2 (train/test Sessions) x 15 (Tap-Stimulus-bins) analysis of variance (2-way
ANOVA), with repeated measures on both factors. This assessed both within-session
development of habituation (learning) and between-session persistence of habituation (memory).

Planned comparisons of the initial (1st bin), middle (8th bin), and final (15th bin) final
phase of stimulation were investigated further for response-differences between training and test
curves. To this end, a one-tailed paired t-test was used because a directional hypothesis (i.e., response-decline) was made. The above analysis procedures were repeated for each response-component (i.e., probability, duration, speed), with the justification that multiple ANOVAs are appropriate when some dependent variables have been studied previously in univariate contexts but are believed to be conceptually independent (Huberty & Morris, 1989; Fugate, Zentall, & Gentry, 2013). Indeed, previous studies of the tap-based reversal have used “magnitude” whereas more contemporary studies have shown that its components – namely, probability, duration, and speed – have different biological underpinnings. A significance threshold of P < 0.05 was used in the analyses of each independent response-component.

**Assessment Across All Response-Components**

To compare within-session habituation (during training) across all response-components, the response values of a plate were normalized as percent initial level. To assess relative differences in within-session habituation a one-way between-subjects ANOVA was conducted on the response emitted within the final (15th) stimulus-bin. A post-hoc Tukey HSD test was used identify pair-wise differences. To assess the relative amount of memory exhibited by each response-component, a difference-score (DS) was then computed for each phase of stimulation. This comprised the arithmetic differences between normalized training and test responses during the initial (1st bin), middle (8th bin), and final (15th bin) phase of stimulation. For example, the following equation was used to compute the memory score for the initial phase of test: \( DS_{\text{initial}} = \text{Train (1st-bin response)} - \text{Test (1st-bin response)} \). For each of these memory-scores, a 1-way between-subjects ANOVA was conducted in order to assess the effect of response-component. Significance level (P-value) was set at 0.05 for ANOVAs.
**Statistical Software**

All data were analyzed using R (version 3.42) and the following packages: tidyverse (Wickham, 2017) and ez (Lawrence, 2016).

### 2.7 Results

Over the course of the training session, all three components of the reversal-response – i.e., probability, duration and speed showed habituation as measured by successively smaller responses (Figure 2). Two-way ANOVAs identified a significant main effect of stimulation (stimulus-bin) for each response-component: probability (F\(_{14,1246}\) = 410.99, p < 0.0001), duration (F\(_{14,1246}\) = 353.84, p < 0.0001), speed (F\(_{14,1246}\) = 117.01, p < 0.0001) [Figure 2]. After the 10-min rest-interval, spontaneous recovery (above habituated levels) was observed in all response-components, as measured by a significant difference between the final response elicited during training and the first response elicited during test (Figure 2): probability [t\(_{18.3}\), df = 89, p < 0.0001], duration [t\(_{23.6}\), df = 89, p < 0.0001, speed [t\(_{2.5}\), df = 89, p < 0.014].

While recovery was observed above habituated levels in all response-components, duration was the only component to show complete recovery to baseline in the 10-min interval. A paired t-test between initial train and test duration showed no significant difference (p = 0.128) [Figure 3]. In contrast, probability and speed showed recovery above the habituated levels, but below baseline level, as evidenced by a significant difference between initial train and test responses (t-test, p < 0.0001). Despite spontaneous recovery to baseline, duration showed evidence of memory later during the test-session, as evidenced by a significant difference between train and test in the middle and final phases of the habituation-curve (p < 0.0001; Figure 3). Thus, memory in the duration component was expressed as faster re-habituation. In contrast, memory in probability and speed manifested as consistently smaller responses during both the
initial phase of the test-session as well as rest of the test-session (P < 0.0001; Figure 3). A significant main effect of stimulation-session (i.e., train/test) was identified for all response-components: probability (F_{1,89} = 189.15, p < 0.0001), duration (F_{1,89} = 435.43, p < 0.0001), speed (F_{1,89} = 501.12, p < 0.0001). In addition, a significant interaction effect between session (train/test) and stimulation (tap-bin) was identified for all response-components: probability (F_{14,1246} = 7.32, p < 0.0001), duration (F_{14,1246} = 9.65, p < 0.0001), speed (F_{14,1246} = 8.04, p < 0.0001). This suggested that the train and test response-curves were not parallel. Indeed, for all response-components the training and test curves approached each other by the end of the test session. In duration, spontaneous recovery to baseline (identified earlier) also clearly contributed to the interaction effect between session and stimulation (i.e., there is no significant difference between the initial response emitted during training and test). Taken together, these data suggest that all three components of the tap-reversal exhibited robust short-term memory for the earlier training; however, they expressed it in different ways or at different phases of the test session.

I next assessed whether there was a systematic relationship between the extent of habituation occurring within the training session and the memory for habituation across the 10-min rest interval. To facilitate comparison across each response-component, amplitude values were normalized to baseline/initial level (Figure 4A). This revealed that the three response-components declined different amounts during training. Most notably, speed underwent the least amount of decline (Figure 4A). This was supported by a one-way between-subject ANOVA on the final (15th) response elicited during training which identified a significant effect of response-component (F_{2, 267} = 282.5, p < 0.0001). A post-hoc Tukey HSD test identified significant pairwise differences between all groups (p < 0.05). Next, to determine whether there were differences in memory among response-components, I computed a memory-score for each phase
of stimulation. This consisted of the arithmetic differences between normalized train and test responses during the initial (1st bin), middle (8th bin), and final (15th bin) phase of stimulation (see Methods for details). Probability and speed showed similar levels of memory across all test-phases (initial, middle, final) [Figure 4B]. In contrast, duration showed little evidence of memory during the initial phase. This was not surprising given that duration showed spontaneous recovery to baseline from training to testing. However, interestingly during the middle phase duration showed more memory than either probability or speed. By the final phase, all response-components showed similar levels of memory Figure 4B). This complex relationship between test-phase and response-component was supported by a 2-way mixed within-subject and between-subject ANOVA. This identified a main effect of test-phase (within-subject factor; F_{2,534} = 11.94, p < 0.0001) but not response-component on memory-score (between-subject factor; F_{2,267} = 2.78, p = 0.063). Interestingly, however, a significant interaction effect between test-phase and response-component was identified (F_{4,534} = 14.42, p = 0.0001) suggesting that the amount of memory shown significantly varied depending on the specific combination of these factors.
Figure 2: Habituation, Spontaneous Recovery, and Re-habitation in N2/wild-type worms during Training and Test Sessions. Values shown are taken from averaging 90 plate-average responses (~40 worms tracked per response per plate; total N ≈ 3600 worms). Error bars (very small) are standard error of the mean (SEM). * denotes significant spontaneous recovery in elicited-response between the end of training (15th stimulus-bin) and beginning of test (16th stimulus-bin) [p < 0.05: paired t-test]. # denotes significant main effects of session (train/test) and tap-bin on response-amplitude for each response-component (Two-way ANOVAs: # p < 0.0001: Probability (F_{14,1246} = 410.99, p < 0.0001), Duration (F_{14,1246} = 353.84, p < 0.0001), Speed (F_{14,1246} = 117.01, p < 0.0001)).
Figure 3: 10-min Habituation-Memory in N2/Wild-Type at Each Phase of the Test-Session in Each Response-Component: The probability, duration, and speed of tap-elicited reversals in N2/wild-type worms both during training-session and during test-session 10-min later: * denotes that the difference in elicited-response between training and test sessions is statistically significant for the corresponding tap-bin assessed (paired t-test, p < 0.0001)
Figure 4: Relative Amount of Within-session Habituation and Between-Session Habituation-Memory Across Response-Components in Wild-Type: The relative change from baseline in the probability, duration, and speed of tap-elicited reversals during the training-session (within-session). Values are equivalent to those in the previous figures (Figure 1 and 2) but are rendered as a percentage from baseline: * indicates a significant effect of response-component on final habituated level (one-way between-subjects ANOVA on the response elicited during the 15th tap-bin). ‘#’ indicates significant differences in final habituated levels between all response-components (post-hoc Tukey HSD test p < 0.05) Values shown are taken from averaging 90 plate-average responses (~40 worms tracked per point, per plate). Error bars are standard error of the mean. & indicates a significant effect of test-phase (i.e., a within-subject predictor) on memory score [p < 0.05; 2-way mixed between- and within- subjects ANOVA].
2.8 Discussion

The reversal-response to tap is made up of three components: probability, duration, and speed. Each component of the reversal-response declined/habituated with repeated tap-stimulation (Figure 2). The proportion of animals that did not respond increased as a result of training (lower probability) and those which did respond, responded less vigorously (smaller duration and slower speed) over the course of the training session. After the 10-min rest interval, response-duration recovered back to baseline levels while probability and speed also recovered but not back to baseline levels. It is interesting to note that although the initial response-duration recovered in the 10-min interval, habituation of response-duration to the test stimuli was more rapid and reached a significantly lower level than in training. At the same time the test curves for probability and for speed were consistently lower than the curves for training (Figure 3). A significant interaction between stimulation (tap-bin) and session (train/test) was identified for all response-components. This suggested that the habituation-curves produced in training and test sessions were not parallel. Indeed, it appeared that the training and test curves might converge with additional stimulation. There are two possibilities: 1) memory diminished throughout the test session, or 2) more likely, this was the result a floor effect. Indeed, Rankin & Broster (1992) found that response-decline in terms of magnitude had reached asymptotic level by the 30th stimulus (i.e., continuing to deliver 30 additional stimuli without a rest-interval did not significantly alter the response). However, in this study, a rest-interval of 10-min occurred between each session of 30 stimuli.

Taken together, these data indicate that each of the three components of the tap-response showed a change in behaviour from training to test, suggesting that each component retained habituation-memory across the rest-interval; however, this memory was expressed in different
ways. Had a single test stimulus been delivered after the rest-interval, it would have appeared that duration had not exhibited any retention (i.e., because duration showed spontaneous recovery to baseline) and yet continued stimulation uncovered evidence of memory. This reinforces the advantages associated with using numerous tests of memory. Even for a simple reflex-like response, multiple tests are required to comprehensively assess the range and persistence of plasticity shown in each of its components.

What is also apparent from dissecting the reversal-response into its components, is that they underwent different amounts of decline with repeated stimulation. Intriguingly, the duration and probability of the reversal-response showed similar kinetics and, converged halfway through the training session (Figure 4A). In contrast, speed showed some facilitation (increase) early in the training session followed by relatively little decline. The speed data are consistent with other instances of habituation which identify some sensitization preceding habituation (Groves & Thompson, 1970).

Given the differences among response-components in their relative decline within the training session, I next tested whether these differences could predict memory – as assessed by the difference-score between training and test curves (normalized) during the initial, middle, and final phase of stimulation (Figure 4). Relative decline within the training-session alone was not a good predictor of memory. Of all the response-components assessed, speed showed the smallest response-decline over the training session. Despite this, it showed remarkably similar levels of memory when compared to probability at all phases of test-session examined (Figure 4B). In contrast, probability and duration showed very similar response-declines within the training session, but sizeable differences in memory across the rest interval both during the initial- and middle-phase of test session. The complex relationship between response-component and test-
phase was supported by an ANOVA which identified a significant main effect of test-phase as well as an interaction effect between test-phase and response-component.

With these data in mind, it is worth hypothesizing an explanation of the apparent dissociation between relative amount of habituation and persistence of habituation. Importantly, this dissociation parallels the relationship between ISI and spontaneous recovery: at shorter ISIs more habituation is observed but spontaneous recovery is also more rapid when compared to longer ISIs which produce less habituation and less rapid spontaneous recovery. Detailed parametric studies suggested that the critical determinant of spontaneous recovery was whether the asymptote characteristic of a specific ISI had been reached (Rankin & Broster 1992). My data reinforces the dissociation between the extent and persistence of habituation but suggests that the dissociation may be observed even at a single ISI among different components of the response. As such, while learning is certainly occurring during the training session, these data together suggest that the relative depth of habituation from baseline both at the aggregate magnitude level and at the finer response-component level does not predict the persistence of habituation (memory). This suggests that the different biological mechanisms known to underlie the habituation curves during training for each response-component (Giles, 2012) might also contribute to the differences in memory within each component. In the search for genetic mechanisms of tap habituation-memory, it may more productive to use different operational criteria for each response-component rather than attempt to fit them into a single metric.
Chapter 3: Genetic Dissection of 10-Minute Tap-Habituation Memory in C. elegans

3.1 Introduction

Thus far, the experiments in this thesis have shown that the probability, duration, and speed of the tap-induced reversal in C. elegans show different amounts of within-session habituation and different amounts of between-session memory (Chapter 2). I have also demonstrated that the response-components may be dissected in terms of the way they express memory. Whereas tap-habituation memory in response-probability and -speed is expressed in terms of spontaneous recovery below baseline, response-duration is expressed in terms of recovery to baseline followed by faster re-habituation (Chapter 2).

This chapter examines whether the differences in memory shown in each response-component (in wild-type) are genetically dissociable using genetic mutations. To this end, candidate mutant strains for study were chosen according to the following criteria: A) the mutated genes had a known role in memory and could, therefore, validate the generality of the tap-habituation memory paradigm (e.g., pde-4/phosphodiesterase-4, acy-1/adenylyl-cyclase-1, and crh-1/CREB). Much of the research in other animal systems has emphasized a critical role for pde-4 and acy-1 in regulating short-term memory but not long-term memory whereas crh-1 is thought to regulate long-term but not short-term memory (Kandel et al., 2014). Therefore, pde-4 and acy-1 (but not crh-1) were hypothesized to show short-term memory deficits in my tap-habituation paradigm; B) other candidate genes included those that were of unique interest to the Rankin Lab because they are expressed in the mechanosensory neurons (e.g., avr-14/glutamate-gated Cl− channel, cmk-1/CAMKI), and C) Additional candidate genes were predicted to show
unique short-term memory phenotypes based on preliminary results from an unpublished genetic screen in the Rankin Lab that investigated 5-min spontaneous recovery from habituation (e.g., cbp-1/CREB-binding-protein, snf-1/GABA-transporter). Collecting two complete habituation curves – one during training and one during the test session – allowed potentiation of habituation to be investigated. That is, habituation that is “more rapid and/or more pronounced” following one or more bouts of spontaneous recovery (Rankin et al., 2009). This paradigm allowed habituation-memory to be observed in a variety of ways besides simply recovery below baseline (as in Figure 1).

Habituation-memory was measured using two scores: 1) difference between the initial response emitting during training and test sessions, and 2) overall (average) response-difference between training and test sessions. By comparing and contrasting how strains of mutant worms scored on these measures, I can detect several forms of habituation-memory. For instance, recovery to below baseline will be detected as a decreased initial-response at test that may or may not be accompanied by an overall (average) response-difference between training and test sessions. If there is no concomitant overall response-difference, then this suggests that memory expression is limited to the initial-response. However, a mutant might also show no initial response-difference but an overall (average) response difference. This would indicate faster re-habituation. Therefore, with two full response-curves (training and test) my paradigm allowed habituation-memory to be characterized more comprehensively than afforded by measures of spontaneous recovery that assess only a single response after a rest-interval.

Another question investigated in this chapter was whether the extent ("depth") of habituation that occurred within the training-session (learning) was predictive of its persistence between-session at test (memory). If supported, this relationship would be useful as it would
suggest that the kinetics of within-session habituation could be used to predict the persistence of habituation at intervals longer than the ISI used during the training-session. This would suggest that the genetic mechanisms underlying the retention of habituation from stimulus-to-stimulus during training also mediate retention at longer time intervals from session-to-session (i.e., training to test). Within the wild-type background, this is not the case because habituation of response-magnitude produced at longer ISIs reaches asymptote more quickly but persists much longer (at least as measured with initial recovery responses: Rankin & Broster, 1992).

However, it is possible that the overall extent of habituation exhibited by a mutant genotype within a single habituation-session (learning) may predict its persistence at a longer interval (memory). In other words, do mutants which show more habituation within a training-session show more persistent habituation-memory? Previous research found that *glr-1* and *eat-4* worms – carrying mutations in genes important for glutamatergic signaling – showed normal if not enhanced 1-hr habituation-memory but deficient 24-hr memory (Rose et al., 2002; see Chapter 1 for detailed review). These examples suggest that the relationship between within-session habituation and between-session persistence of habituation is not straightforward. However, this relationship has not yet been systematically investigated across multiple genotypes in a short-term habituation context.
3.2 Methods

For information regarding strain maintenance, behavior/stimulation regarding tap-stimulation, see Section 2.2. Procedures that are unique to these experiments are described below:

3.3 Strains Phenotyped

Table 2: Mutant Worms Screened for Alterations in Short-term Tap-Habituation Memory. For the complete response profiles for each of these strains during training and testing, see Figure 21.

<table>
<thead>
<tr>
<th>Ortholog/Product</th>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPA receptor</td>
<td>KP4</td>
<td>glr-1(n2461)</td>
</tr>
<tr>
<td>AMPA receptor</td>
<td>VM5519</td>
<td>glr-1(ak10); glr-2(ky176)</td>
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<tr>
<td>NMDA receptor</td>
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<td>nmr-1(ak4)</td>
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<td>NMDA receptor</td>
<td>VC2623</td>
<td>nmr-2(ak3324)</td>
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<tr>
<td>NMDA receptor</td>
<td>VM4343</td>
<td>nmr-1(ak4); nmr-2(ak7)</td>
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<td>avr-14(ad1302)</td>
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<td>Neuroligin</td>
<td>VC228</td>
<td>nlg-1(ok259)</td>
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<td>ztf-11(ok646)</td>
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<tr>
<td>GABA Transporter</td>
<td>RM2710</td>
<td>snf-11(ok156)</td>
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<tr>
<td>GTP cyclohydrolase 1 (Dopamine synthesis)</td>
<td>VC20144</td>
<td>cat-4(gk245686) among many others [strain has been whole-genome sequenced] see MMP website</td>
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<tr>
<td>CREB</td>
<td>YT17</td>
<td>crh-1(tz2)</td>
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<tr>
<td>CBP (CREB-binding protein)</td>
<td>MH24301</td>
<td>cbp-1(ku258)</td>
</tr>
</tbody>
</table>
3.4 Statistical Analyses

Assessment of a Given Response-Component

The “N” for all analyses was number of plates; this is a more conservative enumeration of sample size than either i) the number of worms or ii) number of responses collected per plate. The mean plate-responses to the 60 total stimuli across training and testing were grouped into 30 bins of 2 stimuli each, and the mean of each bin was calculated per plate. These data were then analyzed using a 2 (train/test Sessions) x 15 (Tap-Stimulus-bins) analysis of variance (2-way ANOVA), with repeated measures on both of these factors. This assessed both within-session development of habituation (learning) and between-session persistence of habituation (memory). The above analysis procedures were repeated for each response-component (i.e., probability, duration, speed), with the justification that multiple ANOVAs are appropriate when some variables have been studied previously in univariate contexts (Huberty & Morris, 1989; Fugate et al., 2013). Indeed, previous studies of the tap-based reversal have used “magnitude” whereas more contemporary studies have shown that its components – namely, probability, duration, and speed – have different biological underpinnings (for review, see Chapter 2: Introduction). A significance threshold of P < 0.05 was used in the analyses of each independent response-component.

Statistical Software

All data were analyzed using R (version 3.42) and the following packages: tidyverse (Wickham, 2017) and ez (Lawrence, 2016).

Imputing Missing Values

For several plates, no-reversal response was emitted within a given stimulus-bin (i.e., “a zero response”). This presented a challenge for including these plates in the repeated-measures
ANOVA of the duration and speed of the response (i.e., because data would be missing and, therefore, unbalanced both across and within subjects). To accommodate this, values for the missing duration- and speed-response for the plates were imputed from the preceding or neighbouring responses according to the following rules: 1) If no response was emitted within the final stimulus-bin, then the average of the preceding two responses was taken and replaced the missing value; 2) Otherwise, the missing value was substituted with the average of the neighbouring values. Approximately 12 plates out of a total of 323 plates had one or more values imputed.

**Assessment of Initial Test-Response After Rest-Interval**

A one-tailed paired t-test was conducted between the first tap-stimulus bin of the training and test curves for each response-component. A directional hypothesis (one-tailed) was used since there is precedent that recovery occurs to below baseline (e.g., Rankin & Broster, 1992). A significance threshold of P < 0.05 was used.

**Quantifying Within-Session Development of Habituation Occurring during Training**

The amplitude of the final response elicited within the 15th stimulus-tap bin was subtracted from the first response elicited within the 1st stimulus-tap bin. This method of quantification enabled wild-type to be compared with mutants even if there were differences in initial response-levels (e.g., hyper/hypo-responsive mutants).

**Quantifying Between-Session Persistence of Habituation at Test**

The mean amplitude of the response elicited within the test-session was subtracted from the mean response elicited within the training-session. This method of quantification enabled wild-type worms to be compared with mutant strains even if there were differences in initial response-levels (e.g., hyper/hypo-responsive mutants).
Comparing Within- and Between-Session Habituation Between Mutants and Wild-type

A two-tailed Dunnett’s (many-to-one) test procedure was used to compare both A) the amount of within-session habituation and B) the amount of persistent between-session habituation shown by a mutant with that shown by wild-type. Of note, to control for day-to-day variability in environmental conditions the behavior of each mutant strain was only compared with the corresponding wild-type cohort that was run on the same training/testing days. A two-tailed test was used, given the precedence of both impaired and enhanced learning/memory mutants (i.e., no directional hypothesis). A significance threshold of $P < 0.05$ was used.

3.5 Results

To see the complete response curves for each of the 25 mutant strains during training and testing, consult Figure 21 (Appendix). A summary of the data is shown in Table 1. From left to right, the three main columns show A) the main effect of stimulation (i.e., tap number), B) the main effect of session (i.e., overall differences between training and test), C) interaction between these factors on response-amplitude for each component of the reversal-response for each mutant strain. Green cells indicate a significant effect ($P < 0.05$, *) whereas empty cells indicate no significant effect (2-way repeated-measures ANOVA). From this screen, mutant worms were identified with alterations in within-session habituation and/or between-session persistence of habituation. With the exception of response-speed of $cbp-1$, the response-components in all 25 mutant strains tested showed a statistically significant effect of stimulation (repeated-measures ANOVA, $P < 0.05$; for precise $P$-values and $F$-values see Table 3 and Table 4. This indicated that the response-components for these mutant strains were plastic and their amplitude changed with repeated tap-stimulation.
Many mutant strains were also identified which, like wild-type worms, showed a significant main effect of session, indicating an overall average response-difference between training and test sessions. More interesting, however, was the identification of memory-deficient mutant strains which showed no significant differences (P > 0.05) in the average-response elicited between training and test sessions (white cells in column B of Table 3 and Table 4). Another interesting observation was that wild-type worms showed a significant interaction between the effects of stimulation and session for all response-components. In contrast, all mutant strains showed a loss of this interaction for at least one response-component (white cells in column B of Table 3 and Table 4). The loss of this interaction in mutant strains indicated that the training and test curves for the affected response-component (e.g., speed) are parallel.

Another interesting finding was the identification of mutant strains that showed significant average-differences between training and test sessions in two out of the three response-components but not in the third, which is suggestive of a selective memory lesion (Figure 5): For example, acy-1(GOF), cmk-1, and cdh-4 worms showed selective memory impairments, respectively, in the probability, duration, and speed of the response, while sparing memory in other response-components (Figure 6). Of the 25 mutant strains tested, 9 of them showed no main effect of session for response-probability, 6 of them showed no main effect of session for response-speed and 3 of them showed no main effect of session for response-duration (Figure 5). Interestingly, some mutants showed no significant response-difference between training and test sessions for two out of three response-components. For instance, pde-4 mutant worms showed a significant average-difference between training and test for duration but not for
probability or speed (Figure 5). No mutant was identified that showed a “complete” memory-lesion for all response-components.

Because many past habituation studies have measured memory as the size of the initial response after a rest interval (level of spontaneous recovery), the initial response following the rest-interval was examined more closely. Many mutant strains showed a profile similar to wild-type worms for each response-component: i.e., recovery to below baseline (i.e., significant initial response-difference: $P < 0.05$, one-tailed t-test) for probability and speed but not duration (Figure 7A). However, a variety of mutant phenotypes for spontaneous recovery were also identified in all response-components (Figure 7B). Of the 25 mutant strains screened, about half (13) showed recovery to below baseline for duration, 9 of them showed recovery to baseline for probability, and 3 of them showed recovery to baseline for speed. Comparing initial response-differences with average response-differences (main effect of session) identified mutant strains which showed one memory-measure but not the other across all response-components except for duration (Figure 7). For instance, pde-4 worms showed initial differences in response-speed (recovery to below baseline) but no average difference in response-speed between training and test (main effect of session) [Figure 7B]. In contrast, for duration, no mutant strain was identified that showed a significant initial response-difference but no average response-difference. However, as mentioned above nearly half of the mutant strains screened showed recovery to below baseline for duration unlike wild-type which showed recovery to baseline (i.e., no initial response-difference). Some examples of this reduced recovery phenotype are shown in Figure 9:.

The analyses described above evaluated within-strain differences that occurred as a result of training (i.e., main effects of stimulation, session, as well as initial response-differences). The next question was whether there were A) differences in the amount of within-session habituation
and between-session persistence of habituation shown by a mutant strain compared to that of wild-type worms and B) whether these differences were correlated with one another. A summary of these data is found in Figure 10, with mutant strains that showed alterations in overall within-session habituation colour coded. Across all response-components mutant strains were identified that showed more (coloured red) or less (coloured blue) within-session habituation than wild-type (Figure 10: P < 0.05, two-tailed Dunnett’s test). Sometimes these effects were in the opposite direction when two response-components were examined in a mutant strain. For instance, compared to wild-type, \textit{avr-14} showed smaller within-session habituation for probability but greater within-session habituation for duration (Figure 10).

From this analysis, some mutant strains were identified which showed both greater within-session habituation as well as greater between-session persistence of habituation for probability, as compared to wild-type (e.g., \textit{glr-1}; Figure 10). Likewise, mutants were identified that showed less within-session habituation and correspondingly less between-session persistence of habituation for probability (e.g., \textit{avr-14}; Figure 10). However, many mutants were also identified which showed a less straightforward relationship between within-session and between-session measures of habituation (especially duration and speed, but also for probability). Several mutant strains showed more overall within-session habituation than wild-type worms but which showed equivalent differences to wild-type between average training and test responses (e.g., \textit{avr-14}, duration; Figure 10). Likewise, some mutant strains showed equivalent levels of within-session habituation to wild-type worms but showed either smaller (e.g., \textit{crh-1}, speed; Figure 10, Figure 11:) or larger (e.g., \textit{glr-1}, duration; Figure 10) differences between training and test responses. Linear correlations of within-session habituation with between-session persistence across mutant strains identified no significant relationship (Figure
14). The Pearson $R^2$ correlation-coefficient was consistently below 0.30 in all response-components, suggesting a dissociation between within-session habituation and persistence of habituation. Investigating this in wild-type worms at the plate-level also supported this dissociation (Figure 15). Plates of wild-type worms which on average habituated more within the training-session did not necessarily show greater average differences between training and test curves (Figure 15). The $R^2$ correlation-coefficient was below 0.30 for both duration and speed but essentially 0 for probability ($3.4 \times 10^{-7}$).
Table 3: Candidate Genetic Screen for Mutant Strains Deficient in Short-Term Tap-Habituation Memory

Worms received two sessions of tap-stimulation at 10-sec ISI with a 10-min rest-interval in-between (see Chapter 2 for characterization in wild-type). A 2-way repeated-measures ANOVA was used to determine if there were significant main effects of A) Stimulation (tap number) and B) Session (i.e., train or test) or C) Interaction effects between these factors on response-amplitude for each component of the response (i.e., probability, duration, and speed). The P values for each effect is indicated within the corresponding cell. Green cells indicate a significant effect (P < 0.05, *) whereas empty (white) cells indicate no significant effect (P > 0.05, NS). N = minimum 5 plates per strain. To see the complete response curves for each of these strains during training and testing, consult Figure 21 (Appendix).

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<th>vab-10</th>
<th>cdf-4</th>
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<th>acy-1(LOF)</th>
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**Main & Interaction Effects of Stimulation & Session**

Significance

- p<.05(*): Green cells indicate a significant effect (P < 0.05, *)
- p>.05(NS): Empty (white) cells indicate no significant effect (P > 0.05, NS)

N = minimum 5 plates per strain.
### Table 4: Candidate Genetic Screen for Mutant Strains Deficient in Short-Term Tap-Habituation Memory (F-statistic values).

The data is the same the previous table except F-values are shown in each cell instead of t-values. To see the complete response curves for each of these strains during training and testing, consult Figure 21 (Appendix).

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Figure 5: Genetic Dissection of Short-Term Tap-Habituation Memory into Different Response-Components. Mutant worms highlighted below from the screen showed deficient habituation-memory as measured by a non-significant difference between training and test responses (main effect of session: see previous table) in one or more components of the reversal-response to tap. To see the complete response profiles for each of these strains during training and testing, consult Figure 21 (Appendix).
Figure 6: Genetic and Response-Component Dissection of Short-Term Tap-Habituation Memory. The mutant worms highlighted below showed no significant main effect of session (i.e., memory) for selectively one component the reversal-response to tap (repeated-measures ANOVA between the average train and test responses emitted by plates of worms). ‘**’ indicates P < 0.05. ‘NS’ indicates P > 0.05. For an overview of the precise P-value and F-value consult the previous tables. A) Wild-type/N2 shows a significant main effect of session for all response-components (N = 92), B) acy-1(GOF)/adenylate-cyclase-1 shows no main effect of session for probability (N = 7), C) cmk-1/CaMKI shows no main effect of session for duration, (N = 11), D) cdh-4/Fat-like-cadherin shows no main effect of session for speed (N = 5). To see other mutants with memory-lesions, consult the previous figure.
Figure 7: Comparing Initial Response-Difference (Spontaneous Recovery) with Average Response-Difference (main effect) between Training and Test Curves Across Mutant Strains. A) Wild-type/N2 shows memory as recovery below baseline or recovery to baseline, depending on the response-component (one-tailed paired t-test, \( P < 0.05 \)); however, all response-components show a significant main effect of session (repeated-measures ANOVA, \( P < 0.05 \) indicates a significant difference between the average train and test response). B) Overview of mutant phenotypes on these memory measures. The \( P \) values for each effect is indicated within the corresponding cell. Green cells indicate a significant effect (\( P < 0.05, \ast \)) whereas empty (white) cells indicate no significant effect (\( P > 0.05, \text{NS} \)). To see the F-values, consult Figure 8. \( N = \) minimum 5 plates per strain.
Figure 8: Genetic Dissociation of Initial Response-Difference (Spontaneous Recovery) and Average Response-Difference (Main Effect). A) Wild-type shows memory as recovery below baseline or recovery to baseline, depending on the response-component (one-tailed paired t-test, $P < 0.05$); however, all response-components show a significant main effect of session (repeated-measures ANOVA, $P < 0.05$ indicates a significant difference between the average train and test response) Across various response-components, mutants were identified which showed B) only an initial response-difference but not average response-difference (between train and test curves) C) average response-differences without an initial response-difference (i.e., recovery to baseline) and/or D) neither an initial response-difference nor an average response-difference. ‘*’ indicates $P < 0.05$, ‘NS’ indicates not significant ($P > 0.05$, NS). For an overview of the F-values, consult Figure 8. N = minimum 5 plates per strain.
A Wild-type profile

B initial difference only (no main effect)

C main effect only (no initial difference)

D no main effect & no initial difference
Figure 9: Some Mutant Strains Show Recovery Below Baseline in Response-Duration. A) wild-type shows recovery to baseline [as indicated by a non-significant (NS) difference between the initial response elicited during training and test sessions; one-tailed paired t-test; \( P > 0.05 \)]. B) Some mutant strains show recovery to below baseline (\( P < 0.05 \); see Figure 10B for full list). \( glr-1 \) = AMPA-type ionotropic glutamate receptor; \( mgl-2 \) = metabotropic glutamate receptor. \( avr-14 \) = glutamate-gated chloride channel; \( snf-11 \) = GABA-transporter (orthologous to SLC6A1); \( unc-43 \) = CAMKII; \( N \) = minimum 5 plates/strain.
Figure 10: Wild-type versus Mutant Strain Comparisons of Both Within-Session Development of Habituation and Between-Session Persistence of Habituation. A) The amount of habituation developing within the training session was quantified as the arithmetic difference in response between initial and final response emitted (“depth” of within-session habituation). B) Between-session persistence of habituation was taken as the average response-difference between training and test responses. C) Within-session and between-session measures of habituation were compared between wild-type and mutants (two-tailed Dunnett’s). Red indicates significantly more within-session habituation or between-session persistence compared to wild-type (p < 0.05). To facilitate visualization, difference scores were z-score normalized to the wild-type mean and colour-coded according to the sign and direction of the difference. Blue indicates significantly less within-session habituation or between-session persistence compared to N2 (p < 0.05). White indicates no significant difference between mutant and wild-type.
Figure 11: CREB (crh-1) Mutants Show Intact but Reduced Habituation-Memory in Response-Speed. Worms received a training session and test session – each consisting of 30 taps at a 10-s ISI– separated by a 10-min rest interval. N = 17 (wild-type/N2) and N = 17 (crh-1). Values shown are taken from averaging plate-average responses to tap per genotype (~40 worms tracked per point, per plate) and subsequently averaging the tap-responses into 2-tap bins. Error bars show standard error of the mean. ‘*main effect’ indicates a significant main effect of a stimulation-session repeated-measures ANOVA (p < 0.05: for specific P and F-values, consult Figure 7 and Figure 8) ‘*’ below the initial response denotes that the response-difference between training and test sessions in this stimulus-bin is statistically significant (one-tailed, paired t-test).
Figure 12: Correlating Within-Session Development of Habituation with Between-Session Persistence of Habitation at the Genotype Level. Each strain (wild-type or mutant) is plotted according to how much within-session habituation and between-session persistence it shows. In this paradigm, plates of worms were trained with two sessions of 30 tap-stimuli (delivered at a 10-sec ISI) with a 10-min rest-interval between the sessions. The black line indicates the linear regression line drawn between these variables with the $R^2$ value indicating the strength of the linear association (Pearson correlation coefficient).
Between-Session Persistence of Habituation (Trait_\text{avg} - \text{Test}_\text{avg})

### Probability

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</tr>
<tr>
<td>cbp-1</td>
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</tbody>
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### Duration

- **R^2 = 0.21**

### Speed

- **R^2 = 0.22**

Within-Session Development of Habituation

(Initial_{response} - Final_{response})
Figure 13: Correlating Within-Session Development of Habituation with Between-Session Persistence of Habituation in Plates of Wild-type Worms (at the Plate Level). Each plate of wild-type worms (black dot) is plotted according to how much within-session habituation and between-session persistence it shows. In this paradigm, plates of worms were trained with two sessions of 30 tap-stimuli (delivered at a 10-sec ISI) with a 10-min rest-interval in-between the sessions. The black line indicates the linear regression line drawn between these variables with the $R^2$ value indicating the strength of the linear association (Pearson correlation coefficient).
Between-Session Persistence of Habituation

$\text{(Train}_{\text{avg}} - \text{Test}_{\text{avg}})$

### Within-Session Development of Habituation

**Speed**

- $\Delta$ in mm/sec
- $0.00$, $0.05$, $0.10$, $0.15$
- $R^2 = 0.16$

### Duration

- $\Delta$ in sec
- $0$, $1$, $2$
- $R^2 = 0.28$

### Probability

- $\Delta$ in % worms responding
- $20$, $40$, $60$
- $R^2 = 3.4 \times 10^{-7}$

Plate of wild-type worms
3.6 Discussion

Previous studies have shown that within-session tap-habituation is mediated by distinct genes underlying the probability, duration, and speed of the response (Ardiel, 2012; 2018, Timbers et al., 2013). My data extend these findings and suggest that the persistence of habituation (memory) beyond the training-session in each response-component – as measured by either a) initial response-difference (spontaneous recovery) and/or b) overall (average) response-difference – is also mediated by distinct genes. Mutant strains were identified which showed no initial response-difference (i.e., recovery to baseline) but which showed an overall response-difference (main effect) between train and test sessions. Conversely, mutant strains were identified which showed no significant overall response-difference between sessions but which showed an initial response-difference (i.e., recovery below baseline). Closer examination of the duration of the initial-response at test revealed an enrichment in the number of mutant strains that showed recovery to below baseline (Figure 9): ~50% of mutant strains (13 out of 25) showed this phenotype. The prevalence of this phenotype in the mutant screen was interesting given that wild-type worms showed recovery to baseline. Whereas wild-type worms showed evidence of memory only with continued stimulation (faster rate of re-habituation), the mutant worms described above showed memory immediately (incomplete recovery to below baseline). The prevalence of this mutant phenotype in my screen suggests that spontaneous recovery for response-duration is particularly vulnerable to mutation.

Together, these data suggest that different genetic mechanisms may underlie the phase of test session in which habituation-memory is expressed. Mutant strains were also identified that showed no memory – either in terms of initial response-difference or in terms of an overall response-difference. Intriguingly, some of these “complete” memory lesions were selective for
one response-component while sparing the others. Taken together, this suggests that different genes affect both the phase of memory expression as well as the particular response-component involved.

With respect to overall response-difference (between train and test) across a 10-minute interval, there appeared to be little predictive value afforded by knowing the relative amount of habituation that a genotype exhibited within a training-session (Figure 14). This dissociation between within-session habituation and between-session persistence was further complemented by evaluating the plate-variability in these measures within the wild-type strain (Figure 13). These data suggest that different mechanisms underlie the persistence of habituation across different time-scales – ranging from stimulus-to-stimulus (within-session) and from session-to-session (between training and test sessions).

In many ways, this dissociation between within-session habituation and between-session persistence of habituation is consistent with what is known about the kinetics of ISI-dependent spontaneous recovery in wild-type. With the same number of stimuli, wild-type worms showed less overall within-session habituation at longer ISIs than shorter ISIs (Rankin & Broster 1992) but it persisted longer (as measured by single test responses after various rest-intervals). The critical determinant of recovery was whether asymptote for a given ISI had been reached. If this criterion was not reached then recovery occurred more quickly and was perhaps more sensitive to the number of stimuli delivered (Rankin & Broster 1992). Wicks & Rankin (1996) also demonstrated that laser-ablation of a sensory touch-neuron that contributes to the tap-response (i.e., PLM) decreased within-session habituation without affecting ISI-dependent spontaneous recovery kinetics. This suggested that biologically modifying the tap-circuit in a very targeted way did not alter the persistence of habituation in spite of modifying within-session habituation.
Considering this finding, the memory-specific phenotype shown by mutant strains in my screen may occur due to alterations in the recruitment of ISI-dependent mechanisms.

An unanticipated finding of this screen was that persistent between-session habituation for each component of the reversal-response could be selectively lesioned by genetic mutation. For instance, *acy-1*(GOF), *cmk-1*, and *cdh-4* mutants showed selective memory impairments (as measured by both initial- and overall- response-differences), respectively, for probability, duration, and speed of the response. The specificity of this genetic lesion to persistence (memory) was supported by the fact that within-session habituation (learning) across response-components was relatively preserved, especially in duration and speed. Other noteworthy mutant strains with interesting phenotypes included *crh-1* which showed intact but reduced memory (overall response-difference) for speed and *pde-4* which showed no memory in probability and duration (overall response-difference) and intact but reduced memory (overall response-difference) in speed. All of the genes mentioned above – *acy-1*, *cmk-1*, *crh-1*, *pde-4* – are well-known to be involved in synaptic plasticity and/or memory, and they are all intimately linked with Ca$^{2+}$ second-messenger signalling cascades. A simplified depiction of this signalling cascade is found in
Figure 14 below. What is known about these genes is reviewed below in the following sections.
Figure 14: Simplified Calcium-second Messenger Signaling Cascade: Selected genes identified to be critical for persistent habituation-memory (as measured by both initial response-difference and overall response-difference between train and test curves) in different components of the reversal-response are depicted with response-component affected shown adjacent in a colored box.
**Adenylyl-Cyclase**

Given the range of deficits that mutations in adenylyl cyclase produce in learning/memory paradigms in various organisms, it is no surprise that mutations in the *C. elegans* ortholog of adenylyl cyclase, ACY-1, impaired tap-habituation-memory. What was more intriguing was that the memory-lesion was specific to response-probability for *acy-1*(GOF) but was specific to response-duration for *acy-1*(LOF). A role for adenylyl cyclases in learning/memory has been identified in *Drosophila* in an aversive associative olfactory learning paradigm (*rutabaga*-type) [Dudai, 1988]. These membrane-bound enzymes catalyze the conversion of ATP to cAMP. Within associative paradigms, *rutabaga*-type adenylyl cyclase is often conceived as subserving memory by integrating Ca\(^{2+}\) currents evoked by the conditioned-stimulus (CS) with G protein-coupled activation evoked by the unconditioned-stimulus (US). Since its role in aversive olfactory conditioning was identified in *Drosophila*, adenylyl cyclases have been shown to be critically involved in short-term contextual memory in mice as well as short-term sensitization in *Aplysia* (Wang, Phan, & Storm, 2011; Davis, Cherry, Dauwalder, Han, & Skoulakis, 1995; Hawkins et al., 2006). In *C. elegans*, adenylyl-cyclase-1 (*acy-1*) has been shown to critical for 1-hr appetitive olfactory memory (Stein & Murphy, 2014). Intriguingly, this study demonstrated that both gain-of-function and loss-of-function mutant worms showed reduced memory, suggesting that balanced levels of cAMP is required for normal memory. The distinct memory-phenotypes that *acy-1*(GOF) [probability] and *acy-1*(LOF) [duration] show in my paradigm, suggest that perhaps response-components are differentially sensitive to the balanced levels of cAMP. Other functions that *acy-1* may subserve are basal locomotion and larval growth, as null mutants (*pk1279*) are paralyzed and larval growth is arrested (Moorman & Plasterk, 2002). The
importance of acy-1 is highlighted by the fact that these phenotypes cannot be compensated for by activating other possible adenylyl cyclases through the GSA-1 (Gαs) pathway (Schade, Reynolds, Dollins, & Miller 2005). This makes gsa-1 a promising candidate for assessing a habituation phenotype. In the context of habituation, rutabaga-type adenylyl cyclase has also been shown to be required for short-term habituation-memory of the cleaning reflex in Drosophila (Corfas and Dudai, 1989).

**CREB (cAMP Response Element-Binding protein)**

CREB is a conserved transcription factor known to orchestrate a host of transcription processes that are critical to long-term but not short-term memory. Indeed, the *C. elegans* ortholog of CREB (CRH-1) operates within the interneurons of the tap-withdrawal circuit and its phosphorylation is required for long-term forms of tap-habituation (Timbers & Rankin, 2011; Sugi, Ohtani, Kumiya, Igarashi, & Shirakawa, 2014). Less evidence supports a role for CREB in short-term memory. Indeed, it is hard to envision how CREB-dependent gene expression could affect the 10-min habituation-memory that was produced in my paradigm with only 5-min of training (crh-1 mutant worms showed reduced habituation-memory for response-speed).

However, protein synthesis-dependent depression has been shown in *Helix pomatia* only 6-10 min after a series of EPSPs (Schilhab & Christoffersen, 1996). Protein synthesis has also been shown to be induced only 5 minutes after habituation-training in the HSR paradigm in rats (Murphy et al., 2005, Wright et al., 2006). Moreover, Suzuki et al., (2011) demonstrated that upregulating CREB activity in mice enhanced 30-min memory (contextual fear-conditioning and social recognition task). With a series of pharmacological experiments, it appeared that the enhancement could be accounted for by tonically elevated levels in BDNF shown in mice with
overactive CREB. While BDNF is absent in nematodes, it is likely that CREB regulates the expression of other trophic factors critical to plasticity. Indeed, a binding partner of CREB known as CREB-binding protein (CBP) has been shown to be critical to short-term memory in mice by tonically regulating expression of Ca\(^{2+}/\)CaM-dependent kinase isoforms (including CaMKI\(\gamma\)), and NMDA and AMPA receptor subunits (Chen, Zou, Watanabe, Van Deursen, & Shen, 2010); homologs of these genes exist in *C. elegans* and have been shown to affect between-session habituation-memory (Rose et al., 2003; Lau et al., 2013). Indeed, in my paradigm mutations in the *C. elegans* ortholog of CBP (CBP-1) impaired a) habituation-memory (overall response-difference) in probability and duration and b) rendered response-speed as non-plastic [i.e., response-speed did not change as a result of tap-stimulation]. Considering these findings, it is possible that CBP and CREB together regulate unidentified gene products that are critical for tap habituation-memory in *C. elegans*.

**CMK-1 (Ca\(^{2+}/\)calmodulin-dependent kinase-I)**

Changes in intracellular calcium levels are detected by a variety of sensors including calmodulin (CaM) which, in turn, activate calmodulin-dependent kinases (CaMKs). These kinases transfer phosphates from ATP to a variety of proteins, thereby modifying their location and function. It has been shown that CaMKI plays a role in early-phase long-term potentiation (LTP) in hippocampal cell cultures by means of recruiting AMPA receptors and activating Ras-ERK (Schmitt, Guire, Saneyoshi, & Soderling, 2005; Guire, Oh, Soderling, & Derkach, 2008). Less is known about how CaMKI affects behavioral-plasticity; however, unpublished results in the Rankin Lab demonstrate that the *C. elegans* ortholog of CaMKI (CMK-1) also affects within-session habituation of the reversal-response in a manner that depends on the interstimulus
frequency (Ardiel et al., 2018). Compared to wild-type worms, cmk-1 worms showed a greater habituation in reversal-distance at a 10-sec ISI and much less habituation in reversal-distance at a 60-sec ISI. The results of my genetic screen extend this finding by demonstrating that the effects of CaMKI on habituation extend beyond the training-session. While cmk-1 worms showed robust habituation along all response-components within a training session, they showed poor 10-min habituation-memory in response-duration.

**PDE-4 (Phosphodiesterase-4)**

In *C. elegans*, pde-4 encodes a cAMP-specific phosphodiesterase that cleaves cAMP into 5’AMP. Of the numerous phosphodiesterases encoded within the genome of *C. elegans*, pde-4 is the closest homolog to the human cAMP phosphodiesterase 4D and the *Drosophila dunce* (Charlie, Thomure, Schade, & Miller, 2006). In both associative and non-associative paradigms, dunce mutants show learning and short-term memory defects. Moreover, Shotwell (1983) reported correlations between reduced dunce enzymatic activity and reduced memory. dunce mutants also show more synaptic varicosities in at least one mechanosensory neuron (Corfas & Dudai 1987). Electrophysiological studies show reductions in spontaneous and evoked synaptic currents in dunce mutants as well as reduced synaptic facilitation (Zhong & Wu 1991; Kuromi & Kidokoro 2000). In *C. elegans*, immunostaining suggests that PDE-4 expression is localized to cholinergic and non-cholinergic neurons in the ventral nerve chord (Charlie et al., 2006).

Sequence analysis suggests that the missense mutation carried by *pde-4(ce268)* occurs within the highly conserved catalytic domain and is, therefore, expected to severely reduce enzymatic activity; indeed, an identical amino acid substitution in PDE-5 inhibits cAMP-catalysis by > 8-fold (Turko, Ballard, Francis, & Corbin, 1999). Charlie et al. (2006) also demonstrated that *pde-4*
in *C. elegans* regulates a pool of cAMP that is produced independently of the Gα₃ (GSA-1)-ACY-1 pathway. Indeed, knocking out *pde-4* rescued locomotion deficits in *acy-1* mutants, suggesting that a Gα₃-pool of cAMP was capable of driving locomotion. Moreover, immunohistochemistry in the sublateral nerve chord suggested that PDE-4 was subcellularly focally localized to areas away from synaptic active zones (Charlie et al., 2006). Together, this evidence suggests that the subcellular localization of cAMP (i.e., local concentration) is functionally important. This might help account for the similar but distinct memory phenotypes (in response-component) shown by *acy-1* (GOF) (i.e., probability) and *pde-4* (i.e., probability and speed) in my habituation paradigm.
Chapter 4: Does the Decrement Observed show ISI Dependent Spontaneous Recovery?

3.7 Introduction

The decline in the magnitude of tap-reversals that occurs in *C. elegans* as a result of repeated taps fits the criteria of habituation (see Chapter 1 for review; Rankin et al., 1990; Rankin et al 2009). As described earlier, however, the original aggregate “magnitude” score combined the frequency/probability, duration, and speed of the response. Studies of tap habituation using the Multi-Worm Tracker suggest that the decline in reversal-magnitude to tap can be dissected into three different components (probability, duration, and speed) each of which have distinct genetic underpinnings (Giles, 2012; Ardiel et al., 2018).

This chapter addressed two questions: First, does habituation rather than fatigue and/or sensory adaptation underlie the response-decline observed in a particular component of the reversal response (Figure 2). It is possible that the aggregate response of magnitude fits the criteria of habituation but that one or more of its components (i.e., probability, duration, speed) do not. This is no idle question given the comprehensive phenotypic characterization of mutants provided in the previous chapters. The second question investigated in this chapter was whether or not a given memory-deficit produced by mutation is selective to the ISI of training. Critically, both the development of habituation and the rate of spontaneous recovery are different between short and long ISIs (Rankin & Broster 1992). It is possible that a mutation may impact learning/memory under one training regime but not another.

Because ISI-dependent spontaneous recovery could be used to address both questions – whether the response-decline observed was habituation and whether memory deficits were ISI-
dependent – it was chosen for study. As a reminder, ISI-dependent spontaneous recovery refers to the observation that compared to longer ISIs, shorter ISIs often induce more pronounced but also more persistent response-decline (Rankin & Broster, 1992). This inverse relationship between the extent of response-decline and its rate of recovery is exactly opposite of what would be expected if fatigue or sensory adaptation were chiefly responsible.

My 10-min tap-habituation memory paradigm was modified to investigate this relationship. In addition to training and testing wild-type worms at a 10-sec ISI, two other ISIs were tested: 5-sec ISI and 60-sec ISI. It was hypothesized that all response-components would show ISI-dependent recovery. To assess the possibility that a given mutation may produce a habituation-memory deficit at one ISI but not another, a memory-mutant identified from the screen in Chapter 3 was also trained and tested at 5-sec, 10-sec, and 60-sec ISI. pde-4 mutant worms were chosen for testing because A) they showed robust deficit in probability and speed habituation-memory (as measured by overall response-difference between train and test sessions) and B) the established role that pde-4 (phosphodiesterase) plays in short-term memory across many organisms. If the memory-deficit shown by these mutant worms were ameliorated under different ISIs, then the results would also provide proof-of-principle evidence that performance-deficits (including fatigue/sensory adaptation) did not underlie the response-decline observed in a mutant.

3.8 Method

For general information regarding strain maintenance and behavior/stimulation regarding tap-stimulation see Section 2.2. Procedures which are unique to these experiments are described below:
3.9 Behavior/Tap-Stimulation

Behavioral experiments were conducted approximately 96 hours (4-days) after eggs were laid when worms were considered adults. The MultiWorm-Tracker was used to automatically deliver 30 taps at an interstimulus-interval of 10-sec and collect behavior data. Before tap-stimulation, the Parafilm wax wrapping each plate was removed and the lid of each plate was briefly removed to provide a brief air-puff stimulus to arouse the worms so they could be tracked. 3 minutes after the lifting of the lid tap-stimuli began. Tap-stimulation consisted of a training phase of 30 stimuli delivered at a regular ISI, a 10-minute rest-interval during which no taps were delivered and a test phase of 30 stimuli delivered at the same ISI as delivered during training: therefore, worms received 60 tap-stimuli in total. Worms were randomly distributed into three groups in which they were assigned one of three ISIs: 5-sec, 10-sec, or 60-sec.

Operational Criteria for Tap-elicited Reversal-Response and Worm

As in previous research, tap-elicited reversals were operationally defined as reversals that occurred within 1-second of the tap being delivered. Choreography software was used to pre-analyze data collected by the MultiWorm-Tracker. Only objects (worms) existing for a minimum time of 20 seconds and moving at least 2 body lengths at some point during the experiment were included in analysis in order to exclude artifacts. The time-window over which speed was calculated was 0.1 seconds. To implement these criteria, the following commands were passed to Choreography: -p 0.027 -s 0.1 -t 20 -M 2 --shadowless -S -o nNss*b12xyMmeSakcr --plugin Reoutline::exp --plugin Respine --plugin MeasureReversal::tap::dt=1::collect=0.5::postfix=trv --plugin MeasureReversal::postfix=txt.
3.10 Statistical Analyses

**Assessment of a Given Response-Component**

The “N” for all analyses was number of plates; this is a more conservative enumeration of sample size than either i) the number of worms or ii) number of responses collected per plate. The mean plate-responses to the 60 total stimuli across training and testing were grouped into 30 bins of 2 stimuli each, and the mean of each bin was calculated per plate. These data were then analyzed using two paired t-tests: One t-test was conducted on the initial-response difference given its past and present interest in habituation studies. A second t-test was conducted on the overall (average) response difference between training and test curves. Given that there is precedence that the response-decline may manifest at either level, a directional (one-tailed) hypothesis was made. The above analysis procedures were repeated for each response-component (i.e., probability, duration, speed), with the justification that multiple ANOVAs are appropriate when some variables have been studied previously in univariate contexts (Huberty & Morris, 1989; Fugate et al., 2013). Indeed, previous studies of the tap-based reversal have used “magnitude” whereas more contemporary studies have shown that its components – namely, probability, duration, and speed – have different biological underpinnings. A significance threshold of $P < 0.05$ was used in the analyses of each independent response-component.

**Statistical Software**

All data were analyzed using R (version 3.42) and the following packages: tidyverse (Wickham, 2017) and ez (Lawrence, 2016).
Comparing Habituation Produced by Different ISIs

First, the overall extent of within-session habituation was assessed by conducting a one-way between-subject ANOVA on the average response elicited within the 15th tap-stimulus bin. If a significant effect of ISI was identified, then a post-hoc Tukey HSD was conducted to determine the pairwise differences among the levels of ISI.

To compare the amount of between-session memory that a given ISI produced with that produced by another ISI, the plate-average responses elicited during the test session were subtracted from those elicited during the training session. This response-difference score was then analyzed with a one-way between-subject ANOVA. Significance level (P-value) was set at 0.05 for ANOVAs. When a significant effect of ISI was identified, a post-hoc Tukey HSD test was conducted to determine pairwise differences between scores for each ISI. Significance threshold was set at 0.05.

3.11 Results

Wild-Type Worms

For wild-type worms, changing the ISI affected the overall amount of response-decline shown in any given response-component (Figure 15). For both probability and duration, the shorter the ISI the greater the response-decline. In contrast, an inverse relationship was shown for response-speed in which the longest ISI (60-sec) produced the greatest response-decline (Figure 15). A one-way ANOVA on the final (15th) response supported these observations, showing a significant effect of ISI on amplitude of each response-component: probability: $F_{(2,24)} = 24.4$, $P < 0.001$, duration: $F_{(2,24)} = 10.036$, $P < 0.0001$, speed: $F_{(2,24)} = 5.38$, $P < 0.01$. Post-hoc pairwise comparisons among ISIs showed that probability was the most sensitive to changes in ISI with
all pairwise differences among ISIs being significant (Tukey HSD, P < 0.05). In contrast, for response-duration and speed, only the pairwise differences between the shortest (5-sec) and longest (60-sec) ISI were significant (Tukey HSD, P < 0.05).

Besides affecting within-session response-decline (during training), interstimulus-interval (ISI) also affected the responses emitted during the test-session (Figure 16). For both response-probability and -speed, a significant difference between the initial responses of training and test sessions was observed at all ISIs assessed (one-tailed, paired t-test, P < 0.05; see Figure 16). Interestingly, a different pattern was observed for duration. At both 5-sec and 10-sec ISI, no significant initial response-difference was observed; however, a significant initial-response difference was measured at the longest ISI (60-sec). Investigating the overall habituation curve also showed that the average response elicited during test was smaller than that elicited during training. This main effect of session was identified for all response-components at all ISIs (Figure 16).

To measure memory, the average response-difference between training and test sessions was compared (Figure 17). The difference-scores in response-probability and -speed increased gradually with longer ISIs. In contrast, the difference-scores in duration did not change with ISI. Supporting these observations, a one-way ANOVA identified a significant effect of ISI on difference-scores for probability, and speed but not duration: Probability $F_{(2,24)} = 11.7, P < 0.001$; Duration $F_{(2,24)} = 2.65, P < 0.09$; Speed: $F_{(2,24)} = 9.87, P < 0.001$. Post-hoc pairwise comparisons indicated that the difference-scores in response-probability between the longer ISIs (10-sec and 60-sec) were significantly different [Tukey HSD, P < 0.05] but not between shorter ISIs (10-sec and 5-sec) The opposite pattern was identified for response-duration whereby the difference-
scores between the shorter ISIs (10-sec and 60-sec) were significantly different [Tukey HSD, P < 0.05] but not between longer ISIs (10-sec and 60-sec).

**pde-4 Mutant Worms**

As for wild-type worms, the response-decline shown during training for *pde-4* mutant worms was also largely affected by the ISI used (Figure 18). For both probability and duration, shorter ISIs produced greater response-declines than longer ISIs. Unlike wild-type worms, however, the response-decline for speed in *pde-4* mutants was not affected by ISI. A one-way ANOVA on the final (15th) response supported these observations, showing a significant effect of ISI for probability and duration, but not speed. A one-way ANOVA on the final (15th) response identified a significant effect of ISI for probability and duration but not speed: probability: $F_{(2,22)} = 32.9$, $P < 0.0001$, duration: $F_{(2,22)} = 18.4$, $P < 0.0001$, speed: $F_{(2,22)} = 0.25$, $P = 0.245$. As for wild-type worms, post-hoc pairwise comparisons among ISIs (at the 15th response) showed that probability in *pde-4* mutants was the most sensitive to changes in ISI with significant pairwise differences among all ISIs being significant (Figure 18; Tukey HSD, P < 0.05). For response-duration, however, only the pairwise differences between the shortest (5-sec) and longest (60-sec) ISI were significant (Figure 18; Tukey HSD, P < 0.05). In sum, for both wild-type and *pde-4*, response-decline in duration but especially in probability were sensitive to ISI. In contrast, response-decline in speed in *pde-4* worms was not affected by ISI. One other observation was that across various ISIs and response-components *pde-4* worms often showed less within-session habituation than wild-type worms (Appendix: Figure 24).

In addition to affecting within-session response-decline, ISI also affected the responses emitted by *pde-4* mutant worms during the test-session (Figure 19). For response-probability, a significant difference between the initial-responses of training and test sessions was observed.
only at the longest ISI (60-sec) assessed [Figure 19; one-tailed paired t-test, P < 0.05]. For response-duration, the initial response-differences were not significant for any of the ISIs assessed (P > 0.05). For response-speed, the initial response-differences were significant across all ISIs assessed [Figure 19; one-tailed paired t-test, P < 0.05]. Investigating the overall habituation curves of the 60-sec ISI group also showed that the average response elicited during test was often but not always smaller than that elicited during training. Indeed, response-probability and response-speed showed a significant main effect of session only at the longest ISI (60-sec) assessed [Figure 19; one-tailed, paired t-test, P < 0.05]. This indicated that for response-probability and response-speed, *pde-4* showed memory at longer but not shorter ISIs. Interestingly, at the shorter ISIs of 5-sec and 10-sec, response-probability appeared to show some sensitization between training and test (Figure 19) but the difference was not significant (i.e., no significant main effect of session). In contrast, response-duration showed a significant main effect at all ISIs assessed that manifested as a response-decline [Figure 19; one-tailed, paired t-test, P < 0.05].

Next, the average response-difference between training and test sessions was compared across ISIs and response-components (Figure 20:). Like wild-type, the difference-scores shown by *pde-4* for response-probability and -speed increased with longer ISIs. Unlike wild-type, however, the difference-scores for duration also increased with longer ISIs (Figure 20:). A one-way ANOVA identified a significant effect of ISI on difference-scores for all response-components in *pde-4*: probability: $F_{(2,27)} = 9.54$, $P < 0.001$, B) duration: $F_{(2,27)} = 4.07$, $P < 0.03$, C) speed: $F_{(2,27)} = 9.82$, $P < 0.0001$. Post-hoc pairwise comparisons across all response-components indicated that the difference-scores among ISIs were significant between the longer ISIs (10-sec and 60-sec) but not the shorter ISIs (10-sec and 5-sec) [Tukey HSD, $P > 0.05$;
Figure 20:). In sum, the amount of memory shown by pde-4 worms for all response-components increased at longer ISIs. This ISI-dependent increase in memory occurred for probability and speed (components for which pde-4 showed deficient memory at a 10-sec ISI) as well as duration (a component for which pde-4 showed intact memory at a 10-sec ISI). Of note, although pde-4 worms showed memory for probability and speed at a 60-sec ISI, it was relatively reduced compared to wild-type worms (Appendix: Table 5).
Figure 15: The Effect of Interstimulus-Interval (ISI) on Response-Decline in Wild-type During the Training-Session: A) During training, wild-type worms received a session of 30 tap-stimuli (grouped into 15, 2-tap bins) at a fixed ISI of 5-sec, 10-sec, or 60-sec. B) A one-way ANOVA on the final (15th) response identified a significant effect of ISI for all response-components: Probability: F(2,24) = 24.4, P <0.001, Duration: F(2,24) = 10.036, P < 0.0001, Speed: F(2,24) = 5.38, P < 0.01, ‘*’ denotes significant pairwise differences in the final response (Tukey HSD, P < 0.05). ‘NS’ denotes not significant (P > 0.05). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate). Error bars show standard error of the mean (SEM).
Figure 16: The Effect of Interstimulus-Interval (ISI) on Test-Responses in Wild-type Worms 10-min After Training. As shown in the previous figure, wild-type worms received 30 taps delivered at either 5-sec, 10-sec, or 60-sec ISI during the training session. After a 10-min rest-interval, worms were then re-stimulated with 30 taps delivered at the same ISI they had received during training. Both the initial-response (boxed points) and the overall average-response elicited during training or test session (‘{‘) was analyzed for persistence of response-decline. ‘*’ indicates a significant difference (P < 0.05, paired t-test, one-tailed). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate). Error bars show standard error of the mean (SEM).
Figure 17: Comparing the Response-Differences Between Training and Test Sessions Among Wild-type Worms Trained at Different Interstimulus-Intervals (ISIs). Worms were trained and tested with two sessions of 30 tap-stimuli at one of three ISIs (5-sec, 10-sec, or 60-sec ISIs). Shown below are the average response-difference between training and test curves for groups of worms trained at a given ISI in each response-component. A one-way ANOVA identified a significant effect of ISI on difference-scores for Probability (A), and Speed (C) but not Duration (C): Probability: $F_{(2,24)} = 11.7$, $P < 0.001$, Duration: $F_{(2,24)} = 2.65$, $P < 0.09$, Speed: $F_{(2,24)} = 9.87$, $P < 0.001$ ‘*’ denotes significant pairwise differences in the difference-scores (Tukey HSD, $P < 0.05$). ‘NS’ denotes not significant ($P > 0.05$). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate). Error bars show standard error of the mean (SEM).
Figure 18: The Effect of Interstimulus-Interval (ISI) on Response-Decline in *pde-4* Mutants During the Training-Session: During training, *pde-4* mutant worms received a session of 30 tap-stimuli (grouped into 15, 2-tap bins) at a fixed ISI of 5-sec, 10-sec, or 60-sec. A one-way ANOVA on the final (15th) response identified a significant effect of ISI for probability and duration but not speed: Probability: $F_{(2,22)} = 32.9$, $P < 0.0001$, Duration: $F_{(2,22)} = 18.4$, $P < 0.0001$, Speed: $F_{(2,22)} = 0.25$, $P = 0.245$. ‘*’ denotes significant pairwise differences in the final response (Tukey HSD, $P < 0.05$). ‘NS’ denotes not significant ($P > 0.05$). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate).
Figure 19: The Effect of Interstimulus-Interval (ISI) on Test-Responses in pde-4 Mutants 10-min After Training. pde-4 mutant worms received 30 taps delivered at either 5-sec, 10-sec, or 60-sec ISI during the training session. After a 10-min rest-interval, worms were then re-stimulated with 30 taps delivered at the same ISI they had received during training. Both the initial-response (boxed points) and the overall average-response elicited during training or test session (‘{’) was analyzed for persistence of response-decline. ‘*’ indicates a significant difference (P < 0.05, paired t-test, one-tailed). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate). Error bars show standard error of the mean (SEM).
Figure 20: Comparing the Response-Differences Between Training and Test Sessions Among pde-4 Mutants Trained at Different Interstimulus-Intervals (ISIs). *pde-4* mutant worms were trained and tested with two sessions of 30 tap-stimuli at one of three ISIs (5-sec, 10-sec, or 60-sec ISIs). Shown below are the average response-difference between training and test curves for groups of worms trained at a given ISI in each response-component.

A one-way ANOVA identified a significant effect of ISI on difference-scores in all response-components: A) Probability: $F_{(2,27)} = 9.54, P < 0.001$, B) Duration: $F_{(2,27)} = 4.07, P < 0.03$, C) Speed: $F_{(2,27)} = 9.82, P < 0.0001$ ‘*’ denotes significant pairwise differences in the difference-scores (Tukey HSD, $P < 0.05$). ‘NS’ denotes not significant ($P > 0.05$). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate). Error bars show standard error of the mean (SEM).
3.12 Discussion

The frequency of taps (i.e., ISI) used during training affected both within-session habituation and between-session habituation-memory (as measured by initial response-difference and overall response-difference between training and test sessions). However, the extent of habituation-memory varied depending on a) the response-component and b) the genotype assessed (i.e., wild-type vs. pde-4 worms). In wild-type worms, habituation-memory was less prominent at shorter ISIs – when memory was measured as an initial-response difference at test and as an overall difference-score between training and test. Training at longer ISIs produced smaller declines in response-probability but also produced greater memory (i.e., difference-scores) at test. The decline in response-duration during the training-session was also influenced by the ISI, with longest ISI (60-sec) producing the least decline. At test, while there appeared to be no significant effect of ISI on overall memory (i.e., difference-scores) in response-duration, the only case in which recovery to below baseline (initial-response differences) occurred was with the longest ISI (60-sec). Taken together, the ISI-dependent pattern of decline during training and recovery at test for both probability (overall difference-score) and duration (initial response-difference) are consistent with an underlying habituation mechanism. This partly validates the learning/memory basis of the probability and duration phenotypes outlined in the previous chapters for both wild-type and mutant worms. In other words, there is now evidence to suggest that habituation-alterations underlie these phenotypes rather than sensory adaptation and/or fatigue.

However, unlike probability and duration, speed showed the greatest response-decline at the longer ISI (60-sec). This does not fit the usual pattern of habituation phenomena in which shorter ISIs produce greater response-declines (though there are exceptions to this pattern; see Christoffersen, 1997). However, fatigue and/or sensory adaptation cannot easily explain why
longer ISIs would produce more extensive response-declines. One would expect longer ISIs to produce less response-decline if fatigue and/or sensory adaptation were responsible.

Evaluating response-speed within the framework of Dual-Process Theory may provide a plausible explanation. According to Dual-Process Theory response amplitude is conceived as the net outcome of separate opposing processes – facilitatory and depressing – which are differentially recruited by the intensity, frequency, and identity of a stimulus (Groves & Thompson, 1970). Unpublished results within the Rankin Lab suggest that shorter tap ISIs increase forward speed of worms between taps above baseline (i.e., sensitize) during training whereas longer tap ISIs do not (analogous results have been published for the ASH-induced reversal response: Ardiel et al., 2017); indeed, this was also observed in this experiment (Appendix: Figure 23) whereby sensitization of forward speed was produced with training at 5-sec and 10-sec ISI but not 60-sec ISI. Although no evidence of sensitization of reversal-speed was observed at the behavioral-level it is possible that the underlying facilitatory processes responsible for sensitization of forward-speed are also what prevented the decline in reversal-speed at shorter ISIs. In other words, at longer ISIs, facilitatory processes pertinent to speed were recruited to a lesser extent which thereby enabled the relative contribution of the depressing processes to manifest more robustly in the behavior. This, in turn, led to more within-session decline and between-session persistence of decline (as seen in Figure 15).

One feature of this interpretation, however, that does not cohere well to Dual-Process Theory is the specificity of the conditions determining whether or not sensitization is produced. According to Dual-Process Theory, sensitization arises from general changes in arousal (state) that are not specific to the stimulus/response (Thompson & Spencer, 1970); i.e. it is thought that the facilitatory processes recruited by a given stimulus should increase the response evoked by
other types of stimuli (if not all). That sensitization is only observed in speed but not probability or duration of the reversal-response suggests that the mechanisms underlying sensitization are more specific than predicted by Dual-Process Theory. In support of the selectivity of sensitization, Miller & Domjan (1981) demonstrated that lithium-induced malaise increased aversion to novel gustatory stimuli in rats but not their preference for novel audio-visual stimuli. In contrast, pain by foot-shock increased reactivity to audio-visual stimuli leading to the hypothesis that pain by intrinsic and extrinsic stimulation trigger different sensitization systems.

Another goal of these experiments was to determine whether the memory deficits shown by a strain with a mutation in pde-4 were ISI-dependent. This set of experiments showed that, like wild-type worms, pde-4 mutant worms were sensitive to the ISI both during training and during test. During training, longer ISIs produced the greatest response-declines in probability and duration but not for speed in which ISI had no significant effect (Figure 18). During test, memory deficits in pde-4 mutants were shown in probability and speed (i.e., non-significant overall difference-score between train and test) at the shorter ISIs of 5-sec and 10-sec but not at the longest ISI of 60-sec (Figure 19). Moreover, the difference-scores across all response-components were affected by ISI, with longer ISIs showing greater difference-scores than shorter ISIs (Figure 20). This is intriguing as it suggests that the deficient memory shown by pde-4 mutants in probability and speed are influenced by the particular ISI that is chosen. Importantly, this finding also rules against alterations in fatigue and/or sensory adaptation in accounting for the memory-deficits shown by pde-4 when trained and tested at a 10-sec ISI.

How can one account for this? Literature on long-term memory may provide some answers. An established finding is that whereas massed training is sufficient to produce short-term memory (i.e., consecutive stimulation without rest-intervals), long-term requires spaced-
training (i.e., rest intervals between stimulation sessions) [Wadell & Quinn, 2001]. Longer and more durable forms of memory appear to require greater space between stimulation. In many ways, the relationship between 5-sec ISI and 60-sec ISI is analogous to that of massed-training and spaced-training. To put this in perspective, the duration of the overall training session (delivering 30 taps) for worms stimulated at a 5-sec is ~2.5 minutes whereas it is ~30 minutes for worms stimulated at a 60-sec ISI. It is possible that some of the cellular processes recruited by spaced-training (but not massed-training) are also recruited by longer ISIs in my tap-habituation paradigm. Under this interpretation, the reduced capacity for habituation-memory in response-probability and response-speed in pde-4 may be overcome by the particular training regime. The pro-memory effects of longer ISI, moreover, also increased the amount of habituation-memory that these mutants showed for duration, a response-component in which they were not impaired. At the same time, however, it is important to stress that because the decline in speed during training was not affected by ISI, there is currently no definitive evidence to rule out fatigue and/or sensory adaptation from habituation-deficits in accounting for the speed-phenotype shown by pde-4.

Another analogy that may be worth drawing upon concerns the distinction between long-term potentiation (LTP) and long-term depression (LTD) of synaptic efficacy, both established cellular analogues of learning/memory (Bear & Malenka, 1994). LTP is induced by high-frequency electrical stimulation (HFS) which increases synaptic efficacy between linked neurons in the hippocampus. In contrast, LTD is induced by low-frequency electrical stimulation (LFS) which decreases synaptic efficacy between linked neurons. Given that homosynaptic depression is the favoured mechanism underlying habituation, there are recognizable parallels between LFS and long ISIs in producing a response-decline. From these diverse cellular and behavioral
examples, it is apparent that the temporal properties of stimulus delivery play a key role in memory formation and expression (Kukushkin & Carew, 2017).
Chapter 5: Conclusion

The focus of this project was to characterize a simple memory and investigate its response-components and readiness to genetic dissection. To this end, a granular approach was taken using a high-throughput, high-resolution tracking device that enabled a single reflex-like response to be parsed into three components: probability, duration and speed. Each of these components of the tap-elicited reversal-response in *C. elegans* showed habituation that persisted across a 10-minute interval and expressed memory in different ways. The amount of memory shown in each component did not appear to be directly related to the overall amount of plasticity shown within training (learning). Moreover, neither the amount of habituation-learning or habituation-memory in one component was predictive of the amount of habituation-learning or habituation-memory for any of the other components. This suggested that different genetic mechanisms underlie habituation at different time-scales. In other words, the mechanisms that account for persistent habituation between stimuli delivered in training (within session) are different from those which account for habituation that persists across a longer rest-interval (between session).

The granular characterization of short-term habituation-memory into its response-components was not trivial given that they could, in principle, be genetically dissected from each other. Several genes were identified which, when mutated, selectively impaired habituation-memory in one or more response-component. Initial response-difference and overall-response difference also were genetically dissociated by mutation. Many of the genes identified have been described in the field as being critical to various forms of memory and/or synaptic plasticity. In particular, *acy-1* and *cmk-1* have been implicated in short-term forms of behavioral and/or synaptic plasticity whereas *crh-1* has been implicated in selectively long-term- but not short-term forms. At a course level, my tap-habituation paradigm supported these previous functional-
annotations. Had I operationally defined the reversal-response in terms of magnitude I would have arrived at the same conclusions. However, attending separately to the dimensions of the reversal-response identified subtle forms of memory (specific to response-component) which were spared by genetic perturbation (\textit{pde-4, acy-1 cmk-1}) as well as subtle forms of memory which were unexpectedly altered (\textit{crh-1}). For instance, \textit{acy-1}, though commonly conceived as a short-term memory mutant (e.g., \textit{dunce} in Drosophila), did show deficient habituation-memory in my paradigm but only in response-probability [\textit{acy-1(GOF)}] and response-duration [\textit{acy-1(LOF)}]. Habituation-memory in other response-components was unaffected by these mutations. Likewise, \textit{crh-1} (CREB) though typically thought to be involved selectively in long-term memory consolidation showed reduced short-term habituation-memory in response-speed.

Intriguingly, \textit{pde-4} which is also a canonical gene thought to be involved in short-term memory (i.e., \textit{rutabaga} in Drosophila) showed habituation-memory deficits in response-probability and response-speed at shorter but not at longer ISIs. Together these findings serve as a reminder that a) the level of behavioural analysis chosen is important in determining the functional role a given gene plays in memory: i.e., to what extent a mutation alters memory depends on how the behaviour is operationally defined. b) the demand characteristics of our experimental paradigm sometimes influence to what extent we can detect memory (Tolman & Honzik, 1930), even for simple forms of memory in reflex-like responses. It is intriguing that a simple 10-minute tap-habituation memory is sensitive enough to reveal these subtle effects in genes which are often studied in more complex associative memory paradigms.

Together these data show that short-term memory is not a unitary phenomenon, and that something that affects one aspect of short-term memory may not alter all aspects. To conclude that a mutation, drug, or treatment alters or eliminates short-term memory requires testing
multiple components of a response and multiple training paradigms. This may apply beyond studies of short-term habituation in C. elegans and should be taken into consideration in all studies of short-term memory.

5.1 Future Directions

The dissection of 10-minute habituation-memory into response-components and along genetic lines would greatly benefit from two lines of experiments: 1) stronger validation of the decline in each response-component as genuine instances of habituation; 2) spatio-temporal manipulation of the genes shown to selectively lesion the habituation-memory in each response-component.

With respect to the former this might be achieved by using a dishabituation paradigm. Genetically engineering a strain in which the polymodal nociceptor ASH may be optogenetically stimulated may provide a discrete and precise way to effect dishabituation in each response-component; however, while dishabituation needs to be shown only once in N2 in order to definitively implicate habituation-processes in a response-decline the same cannot be said of mutants. It is possible that genetic perturbation of the tap-withdrawal circuit modifies it such that it is more susceptible to sensory adaptation or motor fatigue. Therefore, the tap-habituation paradigm would benefit from a dishabituation paradigm that can be easily applied to mutant strains. This might be accomplished by delivering blue light in the 350-470 nm range (Ward et al., 2008) which is known to produce negative phototaxis.

To complement this, it would be of great benefit to more definitively identify the causal locus of the target genes implicated in this paradigm. This is because without knocking-down these genes in a spatio-temporally controlled way (in adulthood) it is possible that developmental effects contribute to the deficient memory phenotype. In order to rule out this pleiotropy, inducible constructs could be designed to show that the gene’s effect is on cell function and not a
result of altering development. Alternatively, testing additional alleles would determine to what extent sequence alterations in the gene involved are important for the phenotype.

5.2 Limitations/Caveats & Open-Ended Questions

In a separate project (data not shown), I investigated the effects of delivering an air-puff prior to habituation-training and testing in wild-type worms/N2. Many studies in the Rankin Lab use a behavioral-paradigm that involves delivering an air-puff prior to tap-stimulation. This is done in order to arouse the worms such that they can be detected by the MultiWorm-Tracker. My results suggested that the time between air-puff and tap-delivery had a significant effect on reversal-response. With delays of less than 10 minutes, sensitization of reversal speed (and somewhat reversal probability) was evident: i.e., responses in which the puff-tap delay was less than 10-min were greater than groups in which the puff-tap delay was greater 10-min. With a long puff-tap delay equal or greater than 10-minutes tap-induced sensitization was evident: i.e., the second and third tap elicited a greater response than the first (initial tap). One final observation from this parametric investigation of arousal was that compared to speed and probability, duration showed a small effect of puff during training. Moreover, at test the effects of puff were completely absent for duration except for the initial-response. Invariably, no matter whether the puff:tap delay was 3-min or 10-min the response-duration consistently showed recovery to baseline. It would be interesting to see whether this arousal phenotype in response-duration is affected by mutation in the genes shown to reduce 10-minute recovery to below baseline. In sum, based on many parametric manipulations I found that the tap-habituation memory paradigm is very sensitive to small changes in the protocol.

I also investigated whether there would be differences in phenotype if training and testing with mechanical taps were substituted with direct optogenetic stimulation of the mechanosensory
neurons with channelrhodopsin (Figure 22). As with the tap paradigm worms received two sessions (train and test) each consisting of 30 optogenetic stimuli separated by a 10-min rest-interval. Worms trained with optogenetic stimulation, showed response-declines in all response-components. This provides additional evidence that sensory adaptation cannot account for the response-declines in each response-component (see Chapter 4). However, compared to worms receiving the normal tap, worms which received the optogenetic stimulus showed much faster and deeper habituation in response-probability (Figure 22); other response-components were only subtly affected. The strongest phenotype, however, in optogenetically stimulated worms was in between-session memory. Namely, compared to worms receiving the normal tap, worms that received the optogenetic stimulus showed greater habituation-memory (as measured by both initial-response difference and overall response-difference) in probability and especially in speed. Overall response-differences between these groups of worms for response-duration were comparable; however, worms receiving the optogenetic stimulus showed recovery below baseline response levels. These results suggest that sensory adaptation of tap stimuli may buffer or otherwise prevent habituation from accumulating. According to this hypothesis, when the transduction machinery is bypassed via optogenetic stimulation the mechanisms subserving habituation are recruited more prominently, resulting in both deeper and more persistent habituation. This should be investigated in greater depth in a future study.

The mutant strains of worms I tested might have carried additional (background) mutations beyond the known mutation that could have influenced behavior. To rule this out, either multiple alleles of each gene should be tested or the gene of interest should be rescued or knocked-down in wild-type worms to confirm its role in the phenotype studied. Another issue worth exploring is how to effectively minimize the family-error rate (i.e., the probability of
making one or more type I errors). In this project, memory was quantified by assessing within-strain and between-strain differences in response levels as a result of tap-stimulation. In the case of within-strain comparisons (i.e., response-differences between training and test for a given strain), the p-value was not adjusted for the number of mutant strains tested; however, a Dunnett’s test was used for between-strain comparisons (i.e., response-differences between the wild-type strain and mutant strains) and, therefore, partly addressed the multiple comparisons problem. The Dunnett’s test is designed to minimize the familywise error rate when performing multiple comparisons of treatment group with control. With respect to the statistical analysis, it may also be worthwhile to use regression to analyzing the differences between training and testing habituation-curves. Reducing the differences between training and test habituation-curves to a single line may both facilitate visualization of as well as simplify the statistical tests conducted.
References


The Type 3 Adenylyl Cyclase Is Required for Novel Object Learning and Extinction of Contextual Memory: Role of cAMP Signaling in Primary Cilia


Wagner, A. R. (1981). SOP: A model of automatic memory processing in animal behavior. in N. Spear & R. Miller (eds.), Information processing in animals: Memory mechanisms (pp. 5-47)


Appendix

Figure 21: Training & Test Curves for All Strains in Candidate Genetic Screen. Worms received 2 sessions of tap-stimuli (train & test), delivered at an ISI of 10-sec with a 10-min rest interval in-between. Points correspond to the mean response amplitude emitted by plates of worms (~40 worms per plate). The number of plates trained/tested per strain/genotype is written in the bottom left of each panel (‘n’). Each row of panels corresponds to the amplitude of response in terms of Magnitude, Distance, Probability, Duration, and Speed. Each column of panels corresponds to one strain of worms (e.g., KP4 strain carrying the glr-1 mutation). For statistical analysis regarding initial-difference and overall average-difference between training and test curves consult Table 3 and Figure 7B. The raw data for the screen is separated into 3 strips (A, B, and C). Error bars represent standard error of the mean.
Figure 22: Comparing Habituation-memory Produced by Natural Tap-stimulation with that Produced by Optogenetic-stimulation. Worms received 2 sessions of stimuli (train & test), delivered at an ISI of 10-sec with a 10-min rest interval in-between. N2 (wild-type) and DA1371 (avr-14) were stimulated with natural taps whereas VG88 (wild-type;Pmec-ChR2) and VG545 (avr-14;Pmec-ChR2) were stimulated by optogenetic activation of channelrhodopsin expressed selectively in the mechanosensory neurons. Points correspond to the mean response amplitude emitted by plates of worms (~40 worms per plate). The number of plates assessed per strain/genotype is written in the bottom left of each panel (‘n’). Each row of panels corresponds to the amplitude of response in terms of Magnitude, Distance, Probability, Duration, and Speed. Each column of panels corresponds to one strain of worms. Error bars are standard error of the mean (SEM)
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Figure 23: Forward Speed After Tap at Various Interstimulus Intervals

a) Worms were stimulated with 30 taps at various interstimulus intervals. b) average response-speed forward after tap is delivered is shown. Error bars represent standard error of the mean. N = 8 minimum per group.
Figure 24: Comparing wild-type and pde-4 Worms on Habituation Occurring Within the Training-Session at Various ISIs. Shown below are the average responses shown for groups of worms trained at a given ISI (5-sec, 10-sec, 60-sec) in each component of the reversal-response. ‘*’ indicates a significant difference between wild-type and pde-4 worms on the arithmetic-difference between the initial (1st) and final (15th) response ($P < 0.05$, two-tailed t-test). ‘NS’ denotes not significant ($P > 0.05$). For P-values and F-values, see Table 5 (following page). Values shown are taken from averaging minimum 8 plate-average responses (~40 worms tracked per point, per plate). Error bars show standard error of the mean (SEM).
Table 5: Comparisons of *pde-4* versus wild-type worms on Both Within-Session Development of Habituation and Between-Session Persistence of Habituation at Various ISIs. The amount of habituation developing within the training session was quantified as the arithmetic-difference in response between initial and final response emitted ("depth" of within-session habituation). Between-session persistence of habituation was taken as the average response-difference between training and test responses. Within-session and between-session measures of habituation were compared between *pde-4* and wild-type worms using a two-tailed t-test. The P-value is written in each cell. Blue indicates that *pde-4* worms show significantly less within-session habituation or between-session persistence compared to N2 (P < 0.05), at the specified ISI. White indicates no significant difference between *pde-4* and wild-type worms, at the specified ISI.