Studies in *Caenorhabditis elegans* Olfaction and Learning

By

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Institute of Medical Science

Faculty of Medicine

University of Toronto

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Abstract

Olfactory processes have been extensively studied in the nematode worm *Caenorhabditis elegans*, but much remains unclear regarding both the initial steps of olfactory signal discrimination and components further downstream, in which olfactory memories act to guide behavior. In this work, we identify *lrn-3(mm200)*, a mutant with a broad range of olfactory learning deficits, using a novel screening approach. We then turn to the longstanding question of how worms are able to discriminate between odorants whose signals are transduced via the same secondary messengers, in the same neuron, and show that the apparent discrimination is the result of arrestin-mediated desensitization of odorant receptors. Finally, we show differential signaling by minor G-protein alpha subunits does not play a role in behavioral plasticity after olfactory learning. These experiments demonstrate a rapid technique for identification of learning mutants, and suggest that olfactory discrimination in *C. elegans* may be more limited than previously thought.
Acknowledgements

Thanks foremost to Courtney Lake, without whose encouragement and support I would not have started, to say nothing of concluded, the research described herein. Your curiosity about and knowledge of the natural world are inspirational.

I owe a debt to the students and staff of the van der Kooy lab for their companionship, assistance and helpful discussion. In particular, Brenda Coles-Takabe has, often seemingly miraculously, kept the lab running smoothly, and Jasmina Uzunovic, Celina Tran, Naijin Li and Glenn Wolfe all assisted in performing experiments detailed in this work.

I wish also to express my gratitude to the members of my Program Advisory Committee, Peter McCourt, Peter Roy, Albert Wong and Mei Zhen, for their continued guidance and technical advice.

Finally, thanks to my supervisor, Dr. Derek van der Kooy, for experimental guidance and critique, and for providing an exceedingly stimulating and free environment in which to conduct research.
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Evaluations of PA14 pathogenic bacteria learning behavior (Figure 2) performed by Glenn Wolfe.

Evaluation of isoamyl alcohol / starvation learning (Figure 5) was performed by Jasmina Uzunovic, while that for diacetyl / starvation learning (Figure 6) was performed by Naijin Li. Benzaldehyde / isoamyl alcohol olfactory discrimination assays (Figure 7) were performed by myself and by Celina Tran. Benzaldehyde in a sea of benzaldehyde and AWA olfactory discrimination experiments (Figure 8Figure 9) were performed by Celina Tran. I performed all other experiments.
# Glossary of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AWA, AWC</td>
<td>Amphid Wing Cell A and C</td>
</tr>
<tr>
<td>ARM</td>
<td>Anesthesia-resistant memory</td>
</tr>
<tr>
<td>Bnz</td>
<td>Benzaldehyde</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Chemotaxis Index</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CPEB</td>
<td>Cytoplasmic Polyadenylation Element-Binding</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP Responsive Element</td>
</tr>
<tr>
<td>CREB1</td>
<td>cAMP Responsive Element Binding</td>
</tr>
<tr>
<td>Dia</td>
<td>Diacetyl (IUPAC butanedione)</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethylmethane sulfonate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein coupled Receptor Kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine-5’-triphosphate</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>IsoA</td>
<td>Isoamyl-alcohol (IUPAC isopentyl alcohol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>L1, L2, L3, L4</td>
<td>Larval stages 1, 2, 3 and 4</td>
</tr>
<tr>
<td>LTM</td>
<td>Long-term memory</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>M</td>
<td>Molar (mol/Liter)</td>
</tr>
<tr>
<td>MAP</td>
<td>Mitogen-Activated Protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
</tr>
<tr>
<td>MTM</td>
<td>Medium-term memory</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliters</td>
</tr>
<tr>
<td>NaN3</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>NGM</td>
<td>Nematode Growth Medium</td>
</tr>
<tr>
<td>OR</td>
<td>Olfactory Receptor</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>STM</td>
<td>Short-term memory</td>
</tr>
<tr>
<td>uL</td>
<td>Microliter</td>
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Chapter 1 Literature Review

The 17th century microscopist Robert Hooke remarked that “…many of those Creatures that seem to be very short-lived in respect of Man, may rationally enough be supposed to have lived, and been sensible of and distinguished as many Moments of time as a Man…” (Hooke, 1705) In the centuries since his time, and particularly since the pioneering work of Thomas Hunt Morgan in demonstrating the utility of fruit flies for the study of biology, the value of small model organisms for understanding the principles governing mammalian biology has been demonstrated beyond a doubt. Small, invertebrate model organisms have been found to offer many advantages for (particularly genetic) research: they cost little to maintain, have well-understood, generally short lifecycles and do not pose the ethical problems that experimentation on large vertebrate organisms can. Tellingly, in the century following the first published scientific paper on the genetics of fruit flies (Morgan, 1910), eight Nobel prizes were awarded based on research performed on fruit flies, and another five for research in nematode worms.

Genetic research into the nematode worm *Caenorhabdits elegans* began in the late 1960s, when Sydney Brenner, dissatisfied with the complexity of fruit flies for the study of neural development, sought a simpler model organism to investigate (Brenner, 1974). Having 959 cells in the adult hermaphrodite, a largely invariant cell lineage and very facile genetics, *C. elegans* was quickly recognized as a powerful model by which developmental and behavioral processes might be understood in their entirety. Research in *C. elegans* has been responsible for significant advances in our understanding of aging (Dorman, Albinder, Shroyer, & Kenyon, 1995), embryogenesis (Sulston, Schierenberg, White, & Thomson, 1983) apoptosis (Ellis & Horvitz, 1986) and RNA regulation (Fire et al., 1998), among many others.

Perhaps less obviously, the study of this simple model organism has also provided significant insights into phenomena which might at first appear to be too complex to model in a 1mm long worm, including processing of olfactory information about the surrounding world (Sengupta, Chou, & Bargmann, 1996) and the mechanisms by which learning and memory function (L’Etoile et al., 2002).
Despite the simplicity of *C. elegans*, however, understanding of these processes in worms remains limited.

**Learning and Memory**

Memory is hazy, nebulous and vague. It is the thread which weaves together the stories of our lives, but it is riddled with contradictions: it is alternatingly ephemeral and permanent; it is transient when we most wish it persistent and unaltering when we most wish to forget; it offers clear advantages to its possessor in balance with inscrutably selective capriciousness. Fascination with the often inexplicable workings of memory has motivated great works of literature (Proust, 1913), and intrigued philosophers and scientists for as long as there have been philosophers and scientists.

Plato offers the earliest recorded theory of memory in his “Theatetus”, where he compares memory to a wax tablet in which our experiences and thoughts have been imprinted. The wax substrate of the memory, for Plato, was of different capacities in different individuals, and existed in varying consistencies, such that a firm substrate allowed for longer retention of memory than a soft and more easily smudged substrate. Although subsequent thinkers correctly identified many of the experiential factors impinging on memory formation, including duration, strength and frequency of the stimulus (Locke, 2008), an understanding that neurons formed the physical substrate of memories would wait until the ideas of the philosopher and psychologist David Hartley in the mid-18th century. Hartley’s view that interneuronal signal transduction occurred through vibrations would turn out to be wrong, but in recognizing neurons as broadly being the substrate of memories, Hartley offered the first hints of a concrete account of how, or at least where, memory worked (Glassman & Buckingham, 2007). Claims like Hartley’s were doomed to remain the stuff of parlor conversation until a way could be found to subject them to scientific scrutiny, and this would not occur until the pioneering work of Hermann Ebbinghaus in the 1880s.
Ebbinghaus countered the prevailing view memory in the late 19th century, which was that it was too amorphous a phenomenon to be tractable via experimentation (Murdock, 1985). In a series of systematic experiments using himself as a subject, Ebbinghaus quantified the impact of repeated training sessions on subsequent recall, producing the first learning and forgetting curves, which graphically depicted the gradual development and decline of memory over time. Ebbinghaus’s experimentation finally put the study of memory on a solid scientific footing, but his research demonstrated that the rapidly developing understanding of memory would reveal new conundrums even as it clarified old ones: Ebbinghaus’ experiments revealed the “savings” effect in memory, whereby even after a subject appears to have forgotten something, less subsequent retraining is required to restore their memory than is required to produce a memory of equal strength for the first time. This suggested that some element of memories must remain in place, hidden from access by our conscious minds, long after we lose the ability to access them (Ebbinghaus, 1913).

The idea of a relationship between consciousness and memory was, by this point, already well-established. William James, writing in 1890, had distinguished between “primary memory” (meaning memories which we effortlessly access, without conscious thought) and “secondary memory” (consisting of memories which required active cognition to access) (James, 1890). That distinctions of type existed between various sorts of memories, however, and that these distinctions related in some manner to temporal duration and ease of access, may have been recognized for some time prior to James’ description. Exactly how many types of memory humans have (and even whether there is more than one), and whether we have conscious access to the shortest of them, remains contentious within the field (Cowan, 2008).

Despite this contentiousness, some relatively well-accepted traits of short-term memory in humans have emerged. Among the most historically significant of these resulted from the pioneering work of George Miller, who first proposed a consistent, quantitative limit in the size of working memory. Miller suggested, based on an analysis of data collected by others, that the average person was capable of
maintaining approximately seven items in short-term memory. What constituted an “item”, however, was extremely flexible, and items could be structured to expand the apparent range of short-term memory: Miller suggested that several items could be grouped together in “chunks” which were treated, for purposes of short-term memory limitations, as single items. Whether items could be chunked together into a single item was, for Miller, dependent on whether they made meaningful sense as a single item (Miller, 1956). Subsequent work has proposed reduced extents for the size of working memory (with the reduction, ironically, coming largely from elimination of potential chunking by study participants), but that some limit exists and that chunking plays a significant role in extending it is now well-accepted (Cowan, 2001).

Contemporaneously with Ebbinghaus’ research on himself in Germany, Edward Thorndike was working in the United States, making the first tentative steps toward the development of animal models for studying learning and memory. Thorndike pioneered the use of animals in psychological research by studying the ability of diverse animals, but most famously cats, to solve puzzles for rewards (Thorndike, 1898). By demonstrating that animal experimentation was a viable way to understand learning and memory in humans, Thorndike freed memory research from the ethical limitations of research on humans.

The first testable, cell-mechanistic explanations of memory arrived in 1949, with the publication of Donald Hebb’s “The Organization of Behavior” (Hebb, 1949). Hebb proposed that neurons which repeatedly underwent paired activation would, after time, each begin to facilitate the activation of the others (or as Carla Shatz memorably rephrased it in a later popular publication, “Neurons that fire together, wire together.” (Shatz, 1992)) . Hebb suggested that this alteration occurred through adjustments in synaptic strength (or presence), such that synapses would develop or increase in strength between cells which were reliably activated together, and that the resultant alteration of synaptic connections might constitute the “engram”, or specific biophysical change (or changes) in the brain which was the physical manifestation of a memory.
Although the idea that learning must somehow involve synaptic changes had been proposed long before Hebb (Ramón y Cajal, 1893), his proposal was influential both because offered a testable, easily understood mechanism by which these changes could develop, and because it proposed, for the first time, a plausible account of the precise nature of engrams. Broader searches for engrams had, at this point, been taking place for many years, starting even before the word ‘engram’ had been coined by the German zoologist Richard Semon (Semon, 1921). The first major effort to identify the location of memories in the brain was undertaken by Shepherd Franz, a student of Edward Thorndike’s, who, along with contemporaries, tried to identify the roles of particular regions of the brain via lesion experiments. Franz thought that the frontal lobes might be the substrate of memory, but proved himself wrong in a series of lesion studies on cats and primates (Bruce, 2001; Franz, 1912) which demonstrated a retained ability to retain information even after removal of large portions of the brain. Franz was among the first to concretely demonstrate brain plasticity in response to injury, and perhaps as a result, became an ardent opponent of efforts to localize brain functions to particular neuroanatomical regions (Colotla & Bach-y-Rita, 2002).

Pioneering experiments by Franz’ student, Karl Lashley, taking place over a thirty year span from the early 1920s to 1950, took up this work by seeking to identify the particular brain region response for individual memories in mice and primates, by training animals to associate sensory stimuli with a motor task and then lesioning various parts of the brain. Lashley found that no one particular area of the brain was required for these memories, but rather than the ability of the animals to perform the task after recovery from surgery was best predicted simply by the amount of brain material that had been removed, with removal of diverse regions all causing moderate impairment. On the basis of these experiments, Lashley coined two phrases to describe the neural basis of memory: it was “equipotential”, meaning that no one regions of the brain was more important than any other for memory, and it obeyed “mass action”, meaning that impairment in memory was proportional to the extent of brain injury. Although these ideas
would ultimately be disproved, they exerted a longstanding influence on the field of memory research (Hübener & Bonhoeffer, 2010).

Among the first, and strongest, pieces of evidence suggesting that memory was not equipotential was research by Brenda Milner on the patient then known as H.M. (now known to have been Henry Molaison). Having developed uncontrollably, debilitating seizures following a bicycle accident at a young age, H.M. underwent surgical resectioning of the medial temporal lobes, to which the seizures had been localized, including removal of the majority of the hippocampus. This cured the seizures, but resulted in H.M. developing almost complete anterograde amnesia, which persisted for the remainder of his life (Lichterman, 2009; Scoville & Milner, 1957).

Subsequent research by Milner determined that while H.M. had nearly no memory of events following the operation, and a only a limited memory of events in the 1-2 years immediately preceding it, he showed no performance deficits in tasks unrelated to memory, and was, for the most part, behaviorally unremarkable. Most surprisingly, despite these nearly complete deficits in explicit memory, H.M. was able to learn new motor tasks given moderate practice, although remarkably, he retained no memory of the practice sessions (Milner, Corkin, & Teuber, 1968).

In showing that the hippocampus was crucial for the formation of long-term explicit memories, Milner’s work with H.M. demonstrated that at least some localization of memory was possible, and that different types of memories required different neurological substrates. It is now thought that Lashley’s experiments, focusing on maze learning, required the learning of a complex skill involving the function of a diverse groups of neuroanatomical regions to perform (Josselyn, 2010), and so resulted in an exaggerated view of the equality of brain regions in memory.

Shortly after this, Holger Hydén began undertaking studies attempting to identify the biochemical identity, rather than the neuroanatomical location, of engrams. He observed that the neurons of the lateral vestibular nucleus of the rat increase in RNA content after motor training, and reasoned that RNA, then,
might be the biochemical identity of the memory (Hydén & Egyhazi, 1962). This idea, now discredited, would go on to intersect another line of inquiry in the field, resulting in one of the major setbacks in the history of memory research.

By the early 1950s, frustration was growing with the preponderance of animal studies in experimental psychology making use of rats to the exclusion of other species (Beach, 1950). The potential advantages of using simpler model organisms to study memory were clear, having been demonstrated in the fields of both genetics and development years earlier. Particularly for those researchers attempting to identify a memory engram, the appeal of a small organism, with comparatively few neurons and a more limited set of potential changes which could constitute an engram, were obvious. Adoption of an invertebrate model organism for memory research was hampered, however, by the widespread view that invertebrate animals were incapable of “true” associative learning – that is, that what appeared to be learning was instead a brief instance of behavioral plasticity from which the organism would inevitably soon revert (Maier & Schneirla, 1935), or of non-associative learning being mistaken for associative learning (Jensen, 1965).

The idea that invertebrates were incapable of learning began to crumble with the demonstration in 1955, by Richard Thompson (a former student of Lashley) and James McConnell, of classical conditioning in the planarian flatworm *Dugesia dorotocephala*. In the paradigm initially chosen, Thompson and McConnell showed that training in which the neutral stimulus of light paired with the aversive stimulus of electric shock was sufficient to cause both increased contraction and increased turn rates of the worm in response to future presentation of light alone, while training with electric shock or light alone resulted in no difference from baseline in contraction or turn rates (Thompson & McConnell, 1955). Despite widespread debate about suitable control conditions for the experiment (Rilling, 1996), subsequent investigators successfully replicated these original reports, and confirmed the presence of true associative learning in flatworms (Jacobson, Allan L., Horowitz, Shelder D., Fried, 1967).
Subsequent work by McConnell in characterizing the mechanisms of planarian learning proved less replicable, and remains controversial to this day. Planaria had long been studied for their remarkable regenerative abilities, by which amputation of the majority of the animal results in subsequent healing and complete regeneration of the normal animal organs and body plan (Reddien & Sánchez Alvarado, 2004). This power to redevelop the animal after amputation suggested a powerful technique to localize the engram: animals which had been trained could have large regions of the body amputated, and by identification of the amputated portion which eliminated memories, the anatomical location of the engram might be identified. Exactly this experiment was performed in 1959 by McConnell and collaborators in 1959, checking for a savings effect in regenerated animals upon subsequent retraining to the stimuli. Surprisingly, since the brain and bulk of the nerve tissue is located in the head of planaria, both animals regenerated from head and tail segments exhibited a considerable and approximately equal savings effect on subsequent retraining, suggesting that both regions somehow contained the memory (McConnell, Jacobson, & Kimble, 1959). More surprising still, it appeared that the animals did not even need to derive directly from the same tissues as a trained animal: when planaria which had been trained to a task before being diced into pieces too small to undergo regeneration were fed to naïve planaria which had been starved to induce cannibalism, the naïve planaria also exhibited a savings effect on retraining (McConnell, 1962)!

McConnell then undertook efforts to identify the chemical nature of the engram. Inspired, in part, by Hydén’s findings, he trained planaria to perform a task, before extracting RNA from them and injecting it into naïve planaria (Zelman, Kabat, Jacobson, & McConnell, 1964). Transfer of the RNA alone was found to be sufficient to result in the savings effect, supporting Hydén’s view that engrams were made of RNA (Zelman et al., 1964).

Despite early success replicating of these findings by other labs, and the demonstration that transfer of RNA from trained rats to naïve rats appeared to be sufficient to result in trained responses to sounds (Babich, Jacobson, Bubash, & Jacobson, 1965), doubt soon accumulated among researchers in the
field about the validity of McConnell’s findings (Rilling, 1996; Travis, 1981). Some studies found that only worms regenerated from planaria heads exhibited savings effects when subsequently trained to the task (McConnell, 1966), while many others failed to replicate learning entirely (Weigmann, 2005). Despite occasional recent reports of successful replication of at least some of these findings (Shomrat & Levin, 2013), McConnell’s research is now widely seen as too surrounded by confusion and poor replicability to pursue further (Rilling, 1996).

In the 1970s, three new, quite different invertebrate models were developed, each of which would go on to strongly influence aspects of the field of learning and memory. Working in the sea slug *Aplysia californica*, Eric Kandel’s laboratory was beginning to unravel the neuronal mechanisms of memory by studying simple forms of memory in the gill withdrawal reflex (Castellucci, Pinsker, Kupfermann, & Kandel, 1970). At roughly the same time, work in Seymour Benzer’s lab on *Drosophila* identified the first gene required for learning, *dunce (dnc)*, offering the first evidence that genetic analysis of associative learning processes was feasible (Dudai, Jan, Byers, Quinn, & Benzer, 1976). Simultaneously, in the lab of Sydney Brenner, the first steps in the genetic analysis of the nematode worm *C. elegans* were occurring.

Although the sea slug genus *Aplysia* had been of interest to naturalists going back at least as far as the Roman Empire (Pliny & Jones, 1975), it was not in widespread use as a model system when Erik Kandel began investigating the mechanisms of memory. Kandel came to *Aplysia sp.* after realizing that the complexity of the hippocampus, then being used extensively in memory research, prevented a detailed understanding of the cellular mechanisms of memory (E. R. Kandel, 2007). Working in the laboratory of Ladislav Tauc, who had then been studying *Aplysia sp.* as a model organism for some time, Kandel demonstrated neural facilitation in a particular, consistently identifiable synapse in the animal (E. R. Kandel & Tuac, 1964), demonstrating the utility of the model for understanding synaptic plasticity. Followup experiments, published as a trio of papers, demonstrated habituation and dishabituation of the gill-withdrawal response in *Aplysia sp.* (Pinsker, Kupfermann, Castellucci, & Kandel, 1970), identified their electrophysiological correlates (Kupermann, Castellucci, Pinsker, & Kandell, 1970) and isolated the
underlying changes to a particular synapse in a reduced tissue section (Castellucci et al., 1970). In demonstrating that altered behavioral responses could be reduced to a change in a single synapse, Kandel provided the first strong experimental evidence that synaptic plasticity was the mechanism of at least some forms of memory.

By the mid-1980s, research at Kandel’s lab had largely uncovered many of the molecular mechanisms underlying both non-associative and associative memories in Aplysia sp. Most significantly, formation of long-term memories was found to depend on a signaling cascade in which the secondary messenger cyclic AMP (cAMP) activates protein kinase A (PKA) by causing dissociation of its regulatory subunits. PKA then translocates to the nucleus, where it, along with Mitogen Activate Protein kinase (MAP) kinase, activates cyclic AMP responsive element binding protein 1 (CREB1), which acts as a transcription factor causing increased transcription of genes containing cyclic AMP responsive elements (CREs) in their promoters. The protein products of these genes then mediate the changes in synaptic plasticity (Eric R Kandel, 2012).

Kandel’s research revealed that active mechanisms in memory were not limited to those forming memories, but that cellular machinery also existed to mediate the reverse process. In particular, the transcription factor cyclic AMP responsive element binding protein 2 (CREB2) was found to repress long-term facilitation of synapses, acting in an inverse manner to CREB1 (Bartsch et al., 1995). While CREB1 activation involves phosphorylation by both PKA and MAP kinase, CREB2 deactivation, crucial for derepression at CRE sites, depends only on MAP kinase phosphorylation (Martin et al., 1997).

Memory research in Aplysia sp. benefited greatly from the organism’s large, easily studied neurons, but was limited by the animal not being conducive to genetic manipulation. Conversely when Seymour Benzer came to the fruit fly Drosophila melanogaster in the late 1960s, it had long been a workhorse of genetics, but Benzer’s insight that the facile genetics of the organism might be exploited to allow for analysis of the particular genetic components of behavior was, at the time, both novel and
controversial (Greenspan, 2008). Jerry Hirsch had previously shown that phototaxis in *Drosophila sp.* was under genetic control (Hirsch & Boudrean, 1958), by subjecting groups of flies to strong selection pressure for positive and negative phototaxis, but believed that behaviors must be quantitative traits with no single genes responsible. After identifying a heritable mutation in a chemically mutagenized strain of fly which caused altered phototaxis (Benzer, 1967), Benzer attempted to identify single genes affecting learning, and discovered *dunce* (*dnc*). *dnc*, which encodes a cAMP phosphodiesterase, would soon be shown to be crucial for learning in a variety of other species, including mammals, suggesting that conserved mechanisms of learning exist between comparatively simple model invertebrates and humans, and unambiguously demonstrating the value of behavioral genetic analysis of invertebrates in understanding human biology (Barco, Bailey, & Kandel, 2006).

Subsequent studies in *Drosophila* memory have generally followed the experimental design used by Benzer, in which an odor paired with an electric shock results in subsequent aversion to the odor, although appetitive olfactory learning (in which an odor is paired with sucrose) has also been studied. Experiments soon revealed the existence of dozens of genes playing roles in memory formation and retrieval in flies (Keene & Waddell, 2007). It soon became apparent that both genetic and chemical interventions could dissociate memories in flies into different forms, including short-term memory (STM), anesthesia-resistance memory (ARM) and long-term memory (LTM) (Tully, Preat, Boynton, & Del Vecchio, 1994). Subsequent research revealed still additional forms of fly memory, including three distinguishable phases of anesthesia-resistance memory (Bouzaiane, Trannoy, Scheunemann, Plaçais, & Preat, 2015), and a medium term memory form (Folkers, Drain, & Quinn, 1993). Most remarkably, long-term memory was found to function independently and parallel to short-term memory in flies, such that it is possible to genetically rescue LTM without rescuing any corresponding STM, and vice-versa (Blum, Li, Cressy, & Dubnau, 2009).

Experiments on *Drosophila* are inevitably constrained by its neural complexity and indeterminate neural development, which prevent identification of most types of individual neurons across animals. The
discovery that *C. elegans* was capable of remembering information about its environment, and using this to alter future behavior, offered a solution to this problem.

In the early 1960s, having decided that “nearly all of the ‘classical problems’ of molecular biology have either been solved or will be solved in the next decade”, Sydney Brenner refocused his research from the core problems of molecular biology he had previously been working on to the development and function of the nervous system. Believing that existing model organisms were far too large and complex to allow for the reductionist approach which had been so successful in molecular genetics, Brenner sought a small, rapidly reproducing organism with facile genetics to develop as a new neurodevelopmental model, eventually settling on *C. elegans* (Ankeny, 2001).

After Brenner’s publication of an initial genetic analysis of the animal (Brenner, 1974), researchers spread among an increasing number of laboratories joined the effort. The cell lineage of the embryo was soon worked out (Sulston et al., 1983), followed by the complete connectome of the 302 neurons of the adult hermaphrodite (White, Southgate, Thomson, & Brenner, 1986).

The completely described and determinant neuronal connectivity of *C. elegans*, in conjunction with the biological consistency of its development, offered considerable advantages for research, but may have been responsible for the widespread view that worms were behaviorally “hard-wired” (Rankin, 2004), which perhaps explains why worms were not exploited for the study of memory until the late 1980s. Work by Catherine Rankin first demonstrated that many classical attributes of non-associative memory in other organisms could be demonstrated in worms, including sensitization, habituation and dishabituation, short and long-term memories (Rankin, Beck, & Chiba, 1990) and a link between stimulus frequency and strength of memory formation (Rankin & Broster, 1992).

Much of this early work focused on the “tap withdrawal response”, in which worms respond to a mechanical tap applied to the side of the petri plate they are inhabiting by swimming backward. Other non-associative memories have also been described and studied, including memories for temperature
(Mori, 1999) and habituation to odors (Colbert & Bargmann, 1995). Subsequent research has also revealed more complex forms of memory in *C. elegans*, including associative olfactory memories (Wen et al., 1997), associative humidity memories (Russell, Vidal-Gadea, Makay, Lanam, & Pierce-Shimomura, 2014), occasion-setting (Law, Nuttley, & van der Kooy, 2004), context conditioning (Rankin, 2000) and state-dependent memory (Bettinger & McIntire, 2004). This diversity of memory phenomena in worms includes many of those which have been studied in mammals, providing further support for the use of *C. elegans* as a model organism in which to study memory.

Genetic analysis has, as with most phenomena studied in worms, proven to be a powerful tool by which the mechanisms of memory formation and retrieval can be understood. The first learning mutants identified in worms, *lrn-1* and *lrn-2*, were demonstrated to have a severe deficit in their ability to learn about the (appetitive) paired association of salt and the *Escherichia coli* OP50 bacteria on which they fed, and the (aversive) paired association of salt and garlic extract, without any corresponding deficit in the ability of the worms to respond naïvely to these stimuli, suggesting that the mutation was not effecting a sensory or motor process (Wen et al., 1997). Subsequent research has revealed a variety of other genes required for various learning modalities, including the insulin/IGF ortholog *ins-1*, its receptor *daf-2* (C. H. A. Lin et al., 2010; Tomioka et al., 2006), *easy-1* (Ikeda et al., 2008), *hen-1* (Ishihara et al., 2002), *glr-1* (Rose, Kaun, Chen, & Rankin, 2003), *magi-1* (Stetak, Hörndli, Maricq, van den Heuvel, & Hajnal, 2009) and others.

Olfactory associative memory has been among the best studied memory modalities in worms, which are capable of learning about the pairing of odors with either appetitive or aversive stimuli. Pairing of a neutral or weakly attractive odor with appetitive stimuli (usually a lawn of *E. coli*) causes a subsequent period of increased chemotaxis to that odor (Stein & Murphy, 2014), while pairing of an attractive odor with an aversive stimulus (usually starvation) causes decreased chemotaxis to the odor, or chemotaxis away from the odor (C. H. A. Lin et al., 2010). Whether decreased chemotaxis to the odorant results, or chemotaxis direction is inverted so that it is subsequently away from the odorant, appears to be
dependent on both the odor and the protocol used for training the animals (Tomioka et al., 2006; Tsui & van der Kooy, 2008; Wen et al., 1997). More complex aversive stimuli can also be used for training; in a popular paradigm, the odor of Pseudomonas aeruginosa PA14 is innately more attractive to worms than E. coli OP50, but after exposure to the pathogenic effects of PA14 they learn to eschew it (Yun Zhang, Lu, & Bargmann, 2005).

Among the genes known to play a role in C. elegans learning, loss of function mutations in the insulin/IGF pathway have been shown to result in especially severe starvation association learning deficits for a variety senses, with salt chemotaxis/starvation learning (Tomioka et al., 2006), temperature/starvation learning (Kodama et al., 2006), benzaldehyde/starvation learning (C. H. A. Lin et al., 2010) all being completely or nearly completely eliminated in insulin/IGF pathway mutants. Although there are a plethora of insulin-like peptides in C. elegans, all act via the DAF-2 receptor (Murphy & Hu, 2013) and INS-1 appears to be the major (or perhaps only) insulin-like peptide required for learning in these paradigms. Surprisingly, mutants in the insulin/IGF pathway show enhanced memory in assays of some types of positive associative learning (Kauffman, Ashraf, Corces-Zimmerman, Landis, & Murphy, 2010), leading to the suggestion that this pathway may play a role in transducing the signal for the starvation stimulus (Murphy & Hu, 2013). In benzaldehyde (C. H. A. Lin et al., 2010) and salt (Tomioka et al., 2006) starvation associative learning, INS-1 appears to be released from the AIA interneuron onto the corresponding primary sensory neurons (AWC and ASER, respectively), however corresponding localization of insulin/IGF signaling in temperature/starvation learning has proven challenging (Kodama et al., 2006). In all cases, evidence suggests that INS-1 can act non-cell autonomously to mediate changes in chemotaxis.

As in other organisms, different temporal stages of memory appear to exist in C. elegans. The longest-term memories, both non-associative (Timbers & Rankin, 2011) and associative (Kauffman et al., 2010), appear to be CREB- and spaced-training-dependent, while shorter-term memories are CREB-independent and can be produced by massed training (Stein & Murphy, 2014). To date, unlike in
Drosophila, no mutations are known in C. elegans that selectively affect short-term memory without also causing deficits in long-term memory, and it remains unclear whether long- and short-term memory in the organism is double-dissociable or whether long-term memories are formed as the result of short-term memories.

Significant differences exist in the learning behavior of C. elegans males and hermaphrodites. In paradigms in which starvation is paired with an attractive odor during training, C. elegans males exhibit markedly decreased chemotactic plasticity compared to hermaphrodites, and this has been linked to differences in insulin/IGF-pathway signaling (Vellai, McCulloch, Gems, & Kovács, 2006). Conversely, male worms are able to learn altered chemotactic preferences in response to hermaphrodite proximity, such that previously unattractive tastes become attractive after paired presentation with hermaphrodites – a form of learning with no equivalent in hermaphrodites (Sakai et al., 2013). This sex-specific learning modality requires MCM neurons, only present in the male, which appear to function as locations for sensory integration of cues indicating hermaphrodite presence and of chemosensory signals (Sammut et al., 2015).

Consistent with the discovery of CREB2 in Aplysia, several negative regulators of memory in C. elegans have been identified. EOL-1, a protein implicated in quality control of pre-mRNA, has been identified as a negative regulator of olfactory learning, and loss of function mutants for eol-1 show enhanced Pseudomonas aeruginosa PA14 learning (Y. Shen, Zhang, Calarco, & Zhang, 2014). Other negative regulators appear to play roles after learning has already taken place: animals with mutations in genes in the JNK-1/TIR-1 pathway show reduced forgetting after training, and the requirement for these genes has been localized to sensory neurons (Inoue et al., 2013). A parallel pathway for forgetting has been identified in interneurons, where Arp2/3-mediated changes of the actin cytoskeleton control forgetting (Hadziselimovic et al., 2014).
Olfactory learning in *C. elegans* is usually, but not always, specific to the odorant used in training. Notably, learning to isoamyl alcohol and benzaldehyde cross-adapt in the wildtype animal, with previous training to either one being sufficient to produced trained responses to both (Colbert & Bargmann, 1995).

Notably, many of the genes discovered to play a role in learning in *C. elegans* are critical for some learning modalities but dispensable for others: loss of function mutations in *ins-1*, for example, cause deficits in ability to learn about starvation/odor pairings (C. H. A. Lin et al., 2010) and starvation/salt pairings (Tomioka et al., 2006), but cause no corresponding deficit in learning about pathogenicity/odor pairings (Chen et al., 2013). Even within a single learning modality, however, there is no evidence that screens for key genes have been saturated, and new genes critical for learning are regularly discovered. This slow rate of progress (when compared to other fields of research using *C. elegans*) can be explained both by the complexity of the phenomenon and the laboriousness of the screens.

Across these diverse model organisms, as well as vertebrate models, some common themes of memory formation have emerged. Consolidation of short-term memories to long-term memories depends on the synthesis of new proteins, generally requiring both de novo transcription and translation (Sutton & Schuman, 2006), and is most readily produced through repeated, spaced training, rather than single-session massed training (Bailey, Bartsch, & Kandel, 1996). CREB transcription factors, now known to be conserved among many groups of animals, are responsible for much of the initial protein synthesis during long-term memory (Silva, Kogan, Frankland, & Kida, 1998; Timbers & Rankin, 2011). Despite these commonalities in memory formation, much remains unclear, particularly about the mechanisms by which CREB-mediated transcription resulted in stable, long-term changes in synaptic plasticity.

Much of what is understood about the link between CREB-mediated transcription and long-term changes in plasticity has been discovered only relatively recently. The *Aplysia* protein Cytoplasmic
Polyadenylation Element-Binding protein (CPEB) – a protein previously studied primarily for its role in *Xenopus* development – was found to be induced (probably translationally) after stimulation of serotonergic neurons, and this induction was required for persistence, but not initiation, of long-term facilitation at associated synapses (K Si et al., 2003). Unusually, CPEB was found to have prion-like features, and heterologous expression of CPEB fusions in yeast and bacteria was found to result in heritable, metastable changes in aggregation of the fusion proteins, suggesting a possible mechanism for persistence of synaptic changes (Kausik Si, Lindquist, & Kandel, 2003). More recent studies have shown that the *Drosophila* CPEB ortholog, *orb2*, is required for long-term courtship memories (Keleman, Krüttner, Alenius, & Dickson, 2007), and the *Xenopus* ortholog for visual learning of avoidance behavior (W. Shen et al., 2014).

The role of CPEB in mammalian memory, however, remains far less clear. Mammalian CPEB orthologs include CPEB1, CPEB2, CPEB3 and CPEB4, all of which are expressed in the hippocampus (Theis, Si, & Kandel, 2003). Loss of CPEB1 has only very modest effects on LTP in hippocampal neurons (Alarcon et al., 2004), and causes decreased extinction rates for hippocampal-dependent memories, rather than decreased memory retention (Berger-Sweeney, Zearfoss, & Richter, 2006). Similarly, although CPEB3 does appear to have prion-like characteristics in the brain, dependent on interactions with the actin cytoskeleton (Stephan et al., 2015), deletion mutants exhibit enhanced hippocampal-dependent memory rather than deficits in memory (Chao et al., 2013). No synaptic plasticity or behavioral phenotypes for the remaining two mammalian CPEB mutants, CPEB2 and CPEB4, have been uncovered (Tsai et al., 2013). Although four CPEB orthologs exist in *C. elegans*, no role for them in learning has been identified to date (Rhoads, Dinkova, & Korneeva, 2006).

The longstanding pursuit of memory engrams made significant progress in 2012, with the successful optogenetic reactivation of fear memories by Susumu Tonegawa’s lab. Hippocampal dentate gyrus neurons active during context-specific fear conditioning were labeled with channelrhodopsin-2, and subsequently reactivated outside of the context using light stimulation. Optic activation of these neurons
was sufficient to result in context-independent freezing of the mice, while control mice which had channelrhodopsin-2 labeled dentate gyrus neurons but had not undergone fear conditioning exhibited no freezing, suggesting that reactivation of the dentate gyrus cells active during fear conditioning was sufficient to induce recall of the memory (Liu et al., 2012).

Subsequent experiments built on this result by producing a completely artificial memory in mice – the first molecular creation of a “false memory” in mammals. Mice were exposed to a context without fear conditioning, and active dentate gyrus neurons labeled, after which these neurons were optogenetically reactivated in a second context which was paired with fear conditioning. Subsequent exposure to either the first or the second context resulted in increased freezing behavior, despite the mice only having been fear conditioned in the second context, showing that reactivation of the neurons associated with the first context during fear conditioning had resulted in a memory associating the two being formed (Ramirez et al., 2013).

Most recently, tagged engram cells in the dentate gyrus have been exploited to cast light on the process of memory consolidation. Engram cells were labeled using channelrhodopsin-2 in a fear conditioning task in which some mice received protein synthesis inhibitors (expected to block consolidation of the memory and so prevent its long-term persistence). Although the potentiation of synaptic strength in the labeled neurons of mice which had received the protein synthesis inhibitors was prevented, subsequent optical reactivation of these dentate gyrus neurons was sufficient to restore the fear memory. This reactivation occurred despite other brain regions known to be involved in the memory, including CA3 and the basolateral amygdala, having also been subject to protein synthesis inhibition, and not themselves being reactivated by optic stimulation. Since activation of the engram cells in the dentate gyrus is sufficient, even under protein synthesis inhibition, to reactivate the components of the engram outside the dentate gyrus, these results suggest that protein-synthesis dependent increased synaptic strength is not a component of the long-term memory, although it may be required for its formation (Ryan, Roy, Pignatelli, Arons, & Tonegawa, 2015).
Some limited progress has been made in identifying an engram in *C. elegans*. In the well-studied butanone adaptation olfactory memory paradigm, alterations in levels of cGMP have been shown to cause translocation of the protein kinase G ortholog EGL-4 from the cytosol to the nucleus of the AWCON chemosensory neuron. Nuclear localization of EGL-4 in this neuron is both necessary and sufficient for subsequent behavioral butanone adaptation (He & O’Halloran, 2013; L’Etoile et al., 2002; Lee, 2010; O’Halloran, Hamilton, Lee, Gallegos, & L’Etoile, 2012). Once in the AWCON nucleus, EGL-4 appears to result in upregulation of *odr-1*-derived Small Interfering RNAs (siRNAs), which cause Argonaut-mediated downregulation of *odr-1* mRNA (Juang et al., 2013). *odr-1* encodes a guanylyl cyclase required for chemoattraction to AWCON-sensed odorants, and so its downregulation results in corresponding decreased butanone chemoattraction (L’Etoile & Bargmann, 2000). It is unclear, however, which component of this signaling pathway might constitute the engram, or indeed, if the idea of a single engram is even applicable to this form of memory.

The importance of furthering understanding of the mechanisms of memory is demonstrated by the severe disease burden imposed by disorders of memory. Alzheimer’s Disease, presently the sixth leading cause of death in the United States (Alzheimer’s Association, 2015), is characterized by progressive memory impairment (Selkoe, 2002). Similarly, Parkinson’s Disease manifests with significant memory impairment (Taylor, Saint-Cyr, & Lang, 1990), although intriguingly, memory deficits in Parkinson’s Disease appear to affect recollection of short-term memory and temporal ordering more severely than other memory modalities (Sagar, Sullivan, Gabrieli, Corkin, & Growdon, 1988), suggesting that memory declines in Parkinson’s are not generalized.

In addition to declines in acuity of memory caused by disease, declines in memory performance associated with non-pathological aging alone are well-documented. Memory tasks involving explicit recall of recently learned facts are among the most severely affected, with implicit (procedural) memory being largely spared (Foster, 1999). Evidence from rodents suggests that the mechanism of age-related memory decline may be alterations in capacity for long-term potentiation (LTP) at synapses, consistent
with theories of memory in young animals (Bach et al., 1999; Shankar, Teyler, & Robbins, 1998). This alteration in LTP appears to also be responsible for at least the deficits in spatial memory observed in rodents, and has been localized to the cAMP-protein kinase A-dependent signaling pathway previously shown to be crucial for formation of LTP (Bach et al., 1999). Much about age related memory-decline remains unclear, however, with research in the field being complicated by significant difficulty distinguishing between true cases of age-related memory decline and early stages of memory disorders (Albert, 2002).

**Olfactory Discrimination**

The oldest text in the western world to address the nature of olfaction opens with the claim that “Odours in general, like tastes, are due to mixture.” (Theophrastus, 1916) When Theophrastus wrote this, in approximately 300 B.C.E., he recognized a fact about olfaction that subsequent research would repeatedly validate: nearly all naturally occurring odors consist of a mixture of olfactory molecules, each of which, when present alone, has a distinct smell different from the combination. The smell of a rose, for example, has been shown to consist of a combination of 275 unique component smells (Ohloff, Pickenhagen, & Lawrence, 1994), none of which smell individually identical to the flower as a whole.

For animals, many of which rely on olfaction as one of their most important sensory modalities, accurately finding their way in this mélange of odors is a crucial problem to be solved. With an enormous diversity of odors to be sensed, differing from each other in intensity and quality, olfaction offers an enormously rich source of information to be exploited: even in humans, with olfactory capabilities which are thought to be weak compared to many other species (Castellucci et al., 1970; Rawson et al., 1997), olfactory stimuli are estimated to be over 100,000 fold more discriminable than in sight, the next most sensitive sensory modality (Bushdid, Magnasco, Vosshall, & Keller, 2014) (although this claim is contentious (Gerkin & Castro, 2015)). Conveying this great diversity of information from primary
sensory organs to regions of the brain capable of higher order processing constitutes a challenge for animal signal transduction.

The history of productive scientific research into olfaction is remarkably short, particularly in light of the staggering diversity of researchers who have devoted time to it (Carterette & Friedman, 1977). Much early research was predicated on the idea that, as had been found true of light (Newton, 2012), the great diversity of smells must be reducible to combinations of some very small number of constituent components. No less a classifier than Linnaeus first attempted this, reducing all smells to various combinations of intensities of seven constituents: camphoraceous, musky, floral, pepperminty, ethereal, pungent and putrid (Weinstock, 1985). Various other schemes would follow, including Hendrik Zwaardemaker’s influential late 19th century classification, which expanded the key constituents to nine in number: ethereal, aromatic, fragrant, ambrosiac, alliaceous, empyreumatic, hircine, foul and nauseous (Zwaardemaker, 1889). After the rise and fall of various other classification schemes, this misguided effort reached an apex of influence with the publication in 1927 of the “Crocker-Henderson System”, which claimed to identify four primary smells – fragrant, acid, burnt and caprylic – which, when each ranked on a scale between 1 and 8, were sufficient to unambiguously describe all possible smells (Crocker & Henderson, 1927).

By the late 1960s, a basic picture had begun to form of the physiology of olfaction. The mammalian olfactory epithelium, lining the nasal cavity, was found to consist of olfactory sensory neurons, responsible for the olfactory signal transduction, basal cells, responsible for replenishing olfactory sensory cells, brush cells, responsible for non-olfactory sensory modalities, and supporting cells (Hayran, 2013; Moulton & Beidler, 1967). Olfactory sensory neurons project single, long, unbranched axons through the cribriform plate, and form dense bundles in the olfactory called glomeruli, where they synapse onto the dendrites of mitral and tufted cells (Pinching & Powell, 1971; Read, 1908; Ressler, Sullivan, & Buck, 1994).
A biochemical understanding of the mechanism of olfaction slowly began to arise in the mid 1970’s, with the discovery that binding of odorant molecules to the olfactory sensory neurons occurred in the cilia of the olfactory sensory neurons (Bronshtein & Minor, 1977), and that olfactory signal transduction proceeded through a cyclic AMP (cAMP) dependent mechanism (Pace, Hanski, Salomon, & Lancet, 1985). The subsequent discovery of an olfactory sensory neuron specific G protein alpha subunit, Goif (Jones & Reed, 1989), coupled with biochemical evidence that exogenous guanosine-5’-triphosphate (GTP) further stimulated olfactory cells (Sklar, Anholt, & Snyder, 1986), suggested to some researchers in the field that olfactory receptors (ORs) might constitute of a specialized subgroup of heterotrimeric G-protein coupled receptors (GPCRs). This suggestion was confirmed with the cloning and analysis of representative genes from a diverse family of GPCRs expressed exclusively in the olfactory epithelium (Buck & Axel, 1991). Both the mouse and human genomes were found to contain genes encoding approximately 1,000 olfactory receptors (Ressler et al., 1994; Rouquier et al., 1998), but while nearly all of these genes in the mouse genome are thought to be functional, over 70% of OR-like open reading frames (ORFs) in the human genome are non-functional pseudogenes (Rouquier et al., 1998). The remarkable size of the olfactory receptor gene family makes it the largest gene family in mammalian genomes (Gilad, Bustamante, Lancet, & Pääbo, 2003).

Individual mammalian olfactory sensory neurons were found to express one or very few of the olfactory receptors in the genome (Ngal, Dowling, Buck, Axel, & Chess, 1993; R. Vassar, Ngai, & Axel, 1993). The olfactory epithelium is divided into four zones in mammals, with expression of any particular receptor occurring in a single zone, the location of which is conserved across animals (R. Vassar et al., 1993). Within zones, which receptor or receptors a given olfactory sensory neuron expresses appears to be determined stochastically, with a process of allelic inactivation ensuring that only one receptor from a large array of linked genes is expressed per cell (Chess, Simon, Cedar, & Axel, 1994).

The axons of olfactory sensory neurons expressing the same olfactory receptors converge on the same glomerulus, such that any given glomerulus in the olfactory bulb contains projections from neurons
expressing one, or at most a few, olfactory receptors (Robert Vassar et al., 1994). Activation of a particular olfactory receptor by an odorant, then, results in signal transduction to a particular, corresponding glomerulus. Individual olfactory receptors respond to many odorants, and any particular odorant can stimulate several olfactory receptors, resulting in a combinatorial code for olfaction whereby the identity of the odorant is conveyed to the brain by the particular combination of olfactory receptors activated by it (Malnic, Hirono, Sato, & Buck, 1999). By having each neuron express one, or very few olfactory receptors, and having the axons of these neurons sharing olfactory receptors converge on single glomeruli, the problem of cross-talk between mammalian olfactory receptors is largely avoided. Despite this, the ability of animals to discriminate between odors activating a partially overlapping combinatorial set of glomeruli remains limited, with odors activating highly similar sets of glomeruli being less distinguishable than those activating more divergent sets (Rokni, Hemmelder, Kapoor, & Murthy, 2014).

Although initial studies of the molecular mechanisms of olfaction focused exclusively on agonists of olfactory receptors, researchers have more recently determined that antagonism of olfactory receptors is also likely to play a role in olfactory discrimination (Yuki Oka, Omura, Kataoka, & Touhara, 2004). As might be expected, olfactory antagonists often appear to share structural relationships with agonists, in some cases sharing similar functional groups but with carbon chains of different lengths than in the corresponding agonists (Sanz, Schlegel, Pernollet, & Briand, 2005), or in others being oxidatively dimerized versions of the agonists (Y Oka, Nakamura, Watanabe, & Touhara, 2004). It may be that antagonism by odorants at olfactory receptors, in conjunction with agonism by related odorants, significantly enhances the complexity of the combinatorial olfactory code, allowing for greater olfactory discrimination than could be achieved by a mechanism making use of receptor agonism alone (Sanz et al., 2005).

Although of only limited clinical importance, disorders of olfaction are diverse and common, affecting around one-fifth of the population, and can be significantly detrimental to quality of life. Olfactory disorders are most common as a sequelae of nasal infections and head injuries (Croy, Nordin, &
Hummel, 2014), but can also be genetic in origin, as occurs in the developmental disorder Kallmann Syndrome (MacColl, Bouloux, & Quinton, 2002). Loss of olfaction (anosmia), or reduced olfaction (hyposmia), can be a causative factor in depression, with approximately a quarter to a third of people suffering anosmia being afflicted (Croy et al., 2014).

Failure to detect and discriminate between odors are common in patients suffering from Alzheimer’s disease (Talmo et al., 1989), and have been proposed as one of the earlier diagnostic indicators of the illness (Devanand et al., 2000). Similarly, in Parkinson’s disease, idiopathic hyposmia is among the earlier predictors of the disease (Haehner et al., 2007). The early involvement of the olfactory system in these neurodegenerative disorders has motived the “olfactory vector hypothesis”, by which one or both of these diseases are caused by entry of a chemical or virus through the nasal passage, after which it first affects the olfactory bulb and tract before impacting the remainder of the brain (Hawkes, Shephard, & Daniel, 1999), but the idea remains controversial (Doty, 2008).

Normal variation in human olfactory ability is significant, and cases of specific anosmias have been extensively studied, with variation in ability to the ability to detect the boar pheromone androstenone receiving the greatest attention (Hummel, Krone, Lundström, & Bartsch, 2005). In some cases, the particular receptors underlying the ability to detect these smells have been identified, and single nucleotide polymorphisms or copy number variations in them shown to correlate with the relevant anosmia (Hasin-Brumshtein, Lancet, & Olender, 2009; Keller, Zhuang, Chi, Vosshall, & Matsunami, 2007). Human olfactory sensitivity generally declines with age, with sensitivity to some odors being more severely affected than others (Wysocki & Gilbert, 1989). Diverse explanations have been proposed for these age-related deficits (Kovács, 2004), with the dramatic decline in mitral cells in the olfactory bulb observed in old age being among the most likely causes (Bhatnagar, Kennedy, Baron, & Greenberg, 1987).
Olfaction research in the nematode worm originated with the realization that *C. elegans* exhibited naïve chemotaxis toward or away from volatile odorants (C. I. Bargmann, Hartwig, & Horvitz, 1993), in addition to chemotaxis directed toward water-soluble molecules, which had long been known (Ward, 1973). Over half of the odorants tested by Bargmann et al. were revealed to provoke directed chemotaxis, with most being attractive to *C. elegans*. Chemosensation of several of these odorants was localized to particular cells by laser ablation of individual neurons, and some odorants were found to be sensed nearly exclusively by the AWA, AWB or AWC chemosensory neurons. A wide variety of chemical structures were determined to be sensed by worms, including alcohols, ketones, aromatic compounds and pyrazines. Several mutant strains were isolated which appeared unable to sense some or all odorants, including strains containing mutations in the genes *odr-1, odr-2, odr-3, odr-4* and *odr-5*. Subsequent research has revealed that, in contrast to mammals, worms appear to express many putative olfactory receptors per chemosensory cell – an inevitable result, given that the animal has several hundred putative olfactory receptors and only 32 chemosensory neurons (Bargmann, 2006).

The diversity of chemical structures detectable by worm olfaction prompted Bargmann et al. to ask whether different odorants were distinguishable from each other by worms, or whether their signals exhibited crosstalk, either by sharing a common receptor or by converging on a shared pathway. To resolve this question, an experiment was devised by which worms were placed in a petri dish on agar in which one odorant had previously been uniformly mixed at high concentration, and on which a small point of a second odorant was applied. For many combinations of odorants, worms were found to undergo directed chemotaxis to the point source of odorant, despite being surrounded by a uniform sea of the second odorant. Conversely, when the point odorant was the same as the sea odorant, no chemotaxis to the point was exhibited even if the point odorant was at a significantly higher concentration than the surrounding sea, suggesting that worms could not simply be moving to the point because it smelled more strongly than (but indistinguishably from) the sea. This ability to distinguish between odorants was found
to be the case even for pairs of odorants sensed exclusively by the same cells (such as isoamyl alcohol and benzaldehyde, each sensed by both AWC neurons).

Perhaps most surprisingly, worms were found to be able to distinguish between odorants which both depend on functioning odr-3 (subsequently determined to a G-protein alpha subunit (Roayaie, Crump, Sagasti, & Bargmann, 1998)) and which are sensed exclusively by the same neurons. If loss of odr-3 causes a failure to sense these odorants because it is the alpha subunit associated with the GPCR olfactory receptors responsible for the process, as seems likely, it is unclear how worms are able to distinguish between ODR-3 subunits released from the receptor responsible for sensing one odor, and the same subunits released from the receptor responsible for sensing another. How these olfactory signals are insulated from each other within a cell is an open question in C. elegans research (L'Etoile & Bargmann, 2000).

Subsequent research has shown that, despite worms expressing many olfactory receptors per sensory neuron and mammals expressing only one or very few, much is conserved between olfaction in C. elegans and mammals. In worms, odorant molecules bind to olfactory receptors which line the cilia of amphid sensory neurons. As in mammals, these olfactory receptors are 7-transmembrane GPCRs, although they are not closely related to mammalian olfactory GPCRs (Robertson, 1998). Activation of these receptors results in release of G-protein alpha subunits, which result in downstream signal transduction (Roayaie et al., 1998) via an ion channel. In both groups, this ion channel is cyclic nucleotide gated, although the particular cyclic nucleotide differs: cGMP is used in C. elegans, while cAMP is used in mammals (Komatsu et al., 1999). Indeed, enough of the machinery of olfactory signal transduction is conserved between worms and humans to allow for functional expression of worm olfactory receptors in human cells (Y Zhang, Chou, Bradley, Bargmann, & Zinn, 1997).

Despite these similarities, there are significant differences between mammalian and worm olfaction. Unlike in mammals, the particular sensory neuron olfactory receptors are expressed in plays a
significant role in determining responses to the corresponding ligands. The first olfactory receptor to have an identified ligand in *C. elegans*, ODR-10, was found to be required exclusively for chemoattraction to low-concentrations of diacetyl, with loss of the gene having no effect on chemotaxis toward other odorants (including others known to be sensed by the diacetyl-responsive AWA neuron pair (Sengupta et al., 1996)). Receptors mediating attractive olfactory responses are expressed exclusively in the chemosensory neurons AWA and AWC, while those mediating repulsive responses are expressed in the neurons AWB and ASH (Cornelia I Bargmann & Mori, 1997). When *odr-10* is transgenically expressed exclusively in AWB neurons, diacetyl switches from being chemoattractive to being chemorepulsive, suggesting that the entire valence of odors in *C. elegans* is determined by the identity of the neuron the corresponding receptor is expressed in (Troemel, Kimmel, & Bargmann, 1997). By showing that the valence of the response to chemosensory stimuli depends solely on the cell the corresponding receptor is expressed in, and is independent the identity of that receptor, these experiments raise further questions about how the identity of various ligands binding to a receptors on a single cell can be encoded after initial binding at olfactory receptors.

**Arrestins**

G-protein coupled receptors play diverse and critical roles in mammalian physiology, and constitute the targets of 40% of commercial drugs (Wise, Gearing, & Rees, 2002). Receptors for many neurotransmitters belong to the GPCR family, including serotonin, dopamine, norepinephrine, and some types of GABA and acetylcholine receptors, and play diverse roles in health and disease (Ji, Grossmann, & Ji, 1998). Regulation of the activity of GPCRs is complex, and occurs through diverse mechanisms, with arrestin-family proteins being known to play a central role (Kelly, Bailey, & Henderson, 2008).

Research into arrestins began in 1986, when the key role played by mammalian arrestin-1 in downregulating rhodopsin activity was realized (Wilden, Hall, & Kühn, 1986). Other mammalian arrestins were quickly discovered, including arrestin-2, identified by its regulation of activity of the beta-
adrenergic receptor (Lohse, Benovic, Codina, Caron, & Lefkowitz, 1990), arrestin-3, which regulates synaptic neurotransmitter receptors (Beaulieu et al., 2005; Bohn, Gainetdinov, Lin, Lefkowitz, & Caron, 2000) and adaptation of olfaction (Dawson et al., 1993), and arrestin-4, known to regulate desensitization of cone opsins (Craft & Deming, 2014; Murakami, Yajima, Sakuma, McLaren, & Inana, 1993).

Discovered through a variety of techniques, in diverse cell types, all of these arrestins have subsequently been shown to desensitize one or more GPCRs.

Despite the diversity of arrestin family members, all arrestins operate through a broadly similar molecular pathway. Ligand binding to a GPCR causes it to undergo a conformational change which results in phosphorylation and release of hitherto bound G-protein alpha subunits, and activation of their downstream targets (Neer, 1995). G protein-coupled Receptor Kinases (GRKs), a family of serine/threonine protein kinases, are then able to recognize and selectively phosphorylate these activated GPCRs at residues on their extra-cellular loops or carboxyl terminus. Phosphorylation of the receptor allows the binding of arrestins, which block future binding to G proteins (Kohout, 2003; Reiter & Lefkowitz, 2006). Subsequent phosphorylation of the bound arrestin can cause it to act as a clathrin adapter, resulting in endocytosis of the receptor/arrestin pair in clathrin coated vesicles (F.-T. Lin et al., 1997).

More recently, G-protein-independent cell-signaling roles for arrestins have been identified. The receptor tyrosine kinase family Insulin-like Growth Factor 1 (IGF-1) Receptor was found to be internalized via clathrin-coated pits in a manner dependent on arrestin-2, allowing for subsequent signaling through mitogen-activated protein kinase 1 and 3 (MAPK1 and MAPK3) (F. T. Lin, Daaka, & Lefkowitz, 1998). Later research revealed that arrestins can act as scaffolding proteins, linking receptors (including both GPCRs and non-GPCRs) to non-receptor tyrosine kinase family members. As was the case for the IGF-1 receptor, this scaffolding role allows for downstream activation of MAP kinase-dependent signaling pathways (DeWire, Ahn, Lefkowitz, & Shenoy, 2007; Luttrell et al., 1999). Mutational analysis has revealed that the G-protein-dependent signaling mechanism of at least some
receptors can be completely uncoupled from the arrestin-dependent signaling mechanism (Wei et al., 2003).

Arrestin signaling in worms appears to have fewer components than in mammals, with only a single arrestin ortholog, arr-1, presently known (Palmitessa et al., 2005). arr-1 is expressed widely in the C. elegans nervous system, including in the chemosensory neurons AWA, AWB, AWC, ADL and ASH, and in the HSN neuron (known to be a site of G protein-dependent serotonin reception). arr-1 shows greatest sequence similarity to the lone Drosophila non-visual arrestin kurtz, and to the mammalian non-visual arrestins arrestin-2 and arrestin-3. Worms with loss of function mutations in arr-1 show no deficit in naïve chemoattraction to odorants, but exhibit partial defects in subsequent olfactory adaptation similar to those seen in mouse olfactory neuron cilia deficient in arrestin-3. Conversely, arr-1 mutants also showed deficits in recovery from olfactory adaptation after removal of odorants. Intriguingly, recovery after adaptation, but not adaptation itself, is dependent on the C-terminal region of arr-1: rescues performed with C-terminal truncations fully restore adaptation, but only result in a slight, partial rescue of recovery of olfactory adaptation. Since the C-terminal region of arr-1 contains putative binding motifs for beta-2-adaptin and clathrin, known to be key players in arrestin-mediated endocytosis, and biochemical experiments indicated that these proteins bound to the C-terminal end in vitro, this suggests that arrestin-mediated endocytosis is important in reversing the effects of arrestin desensitization of GPCRs in C. elegans (Palmitessa et al., 2005). This supports findings from previous in vitro and cell culture experiments suggesting that endocytosis of arrestin desensitized GPCRs is required for resensitization to occur (Krueger, Daaka, Pitcher, & Lefkowitz, 1997).

The family of GRKs in C. elegans contains two predicted members, Ce-grk-1 and Ce-grk-2, the latter of which is most closely related to the mammalian GRK known to be required for olfactory adaptation, grk-3 (Fukuto et al., 2004; Peppel et al., 1997). Unusually, although ARR-1 has been found to regulate C. elegans olfactory adaptation in similar ways to arrestin-3 in mammals (Dawson et al., 1993; Palmitessa et al., 2005), loss of function mutation in grk-1 or grk-2, the putative upstream components of
the pathway, do not result in similar olfactory adaptation deficits. Loss of function mutations in \textit{grk-2} instead result in severe olfactory deficits to a broad suite of odorants, suggesting an \textit{arr-1}-independent role for \textit{grk-2} (Fukuto et al., 2004), while loss of function mutations in \textit{grk-1} result in no olfactory deficits (Fukuto et al., 2004) but instead result in subtle alterations in dopaminergic signaling (Wani et al., 2012). \textit{grk-2} mutants exhibiting no non-chemosensory motor defects, and the olfactory deficits present in \textit{grk-2} mutants being rescued in a dose-dependent manner by cell-specific rescue of \textit{grk-2}, suggest that the gene is likely acting on the chemosensory receptors instead of in a downstream neuron more broadly required for coordinated motor movement. It remains unclear how, in the absence of the GRK tagging system used in mammals, worm arrestins select particular GPCRs to be targeted for downregulation (Yamada, Hirotu, Matsuki, Kunitomo, & Iino, 2009).

Some evidence exists for a G-protein-independent signaling role for arrestin in worms. \textit{arr-1} loss of function mutants were found to exhibit increased lifespan, leading to the discovery that ARR-1 acts to positively regulate the canonical lifespan-determining DAF-2 pathway by inhibiting DAF-18 in a complex with MPZ-1 (Palmitessa & Benovic, 2010), despite none of the known effected components in the pathway being GPCRs. This suggests that, as in mammals, arrestin in worms has G-protein-independent roles, but the biochemical mechanism of this effect remains poorly understood.

Defects in visual arrestin (ARRESTIN-1) signaling have been implicated as a cause of Oguchi disease (a rare form of night-blindness), while double-knockouts of \textit{arrestin-2} and \textit{arrestin-3} are known to be embryonic lethal in mice (Pierce & Lefkowitz, 2001). Arrestin signaling has been implicated in asthma (Hollingsworth et al., 2010), immune responses (Shi et al., 2007), cancer (Buchanan et al., 2006), multiple sclerosis (Ohguro et al., 1993) and diverse other diseases.
Chapter 2 Research Aims and Hypothesis

Despite extensive study of the neurobiology of C. elegans, understanding of the processes involved in olfactory signal transduction and behavioral plasticity remains limited. The aims of this work are to identify genes involved in mediating behavioral plasticity, and to clarify the confusing nature of olfactory discrimination in the nematode worm.

The hypothesis underlying the portion of this work on behavioral plasticity is that iterative selection from a pool of randomly mutagenized worms can identify learning mutants far more rapidly than screens of populations derived from individual F2 progeny of mutagenized parents.

The motivating hypothesis for the second chapter of this work is that the seemingly dramatic ability of worms to discriminate between two odors sensed within a single neuron results from arrestin-mediated desensitization of the receptors corresponding to one of these odors. In this hypothesis, what appears to be a qualitative difference in downstream signaling caused by the odors is actually only a quantitative difference.
Chapter 3 Isolation and Analysis of a *Caenorhabditis elegans* mutant with a broad suite of learning deficits

*Introduction*

Modalities of learning in *C. elegans* vary in their ease of reproducibility, the amount of specialized equipment required to study them and the amount already known about their molecular mechanisms. Benzaldehyde, sensed by the paired chemosensory AWC neurons (C. I. Bargmann et al., 1993), was among the first odorants used to study olfactory/starvation associative learning in *C. elegans* (L’Etoile & Bargmann, 2000). Pre-exposure to benzaldehyde under starvation conditions reliably results in the subsequent reversal of chemotactic response to the odorant, such that naïve worms (exposed to starvation alone) are strongly chemoattracted to benzaldehyde, while worms which have been pre-exposed to starvation and benzaldehyde show strong chemorepulsion to it (C. H. A. Lin et al., 2010). The consistently reproducible but incompletely understood nature of this response make it an ideal subject for genetic analysis.

Worms which have learned an altered response to a stimulus do not exhibit this response with complete consistency, with only the majority of worms tested showing the trained response. Naïve responses to odorants similarly exhibit some degree of variability, with occasional naïve worms exhibiting a “trained” response, and both naïve and trained responses vary between trials of single worms rather than between animals, suggesting that the inconsistency is not the result of developmental or genetic differences (Wen et al., 1997). To accommodate this variability in response, genetic screens have often been performed on large numbers of genetically identical worms, with the average response of the animals tested recorded, but these screens are very laborious.

We wondered whether an approach that exploited repeated, iterative selection for mutant worms failing to undergo chemotaxis away from benzaldehyde even after training (presentation with paired
exposure to benzaldehyde and starvation) might allow for easier isolation of learning mutants than traditional screens of clonal populations. Such an approach was expected to select for worms which consistently approached benzaldehyde after training while progressively eliminating worms which happened to approach it by chance, without the need to study large clonal populations of mutagenized worms limiting the number of genotypes that could be screened.
Materials and Methods

Nematode propagation

*C. elegans* were grown in petri dishes on Nematode Growth Media (NGM) and fed OP50 strain *Escherichia coli* (Wood, 1988). All experiments used well fed young adults (52 h) cultivated at 20 °C. When necessary, animals were synchronized by hypochlorite treatment according to standard methods (Stiernagle, 2006). Nematode strains were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota (St. Paul).

Nematode strains

*C. elegans* strains used in this work were: wild type *C. elegans var Bristol* (N2) (Brenner, 1974), *ins-1(nr2091), mIs12(II)[myo-2p::GFP + pes-10p::GFP + F22B7.9p::GFP] (PD4790)* (Edgley, Mark; Liu, Jun Kelly; Riddle, Don; Fire, 1999) and *lrn-2(mm99)* (UT2) (Wen et al., 1997). *C. elegans* strains produced in this work were: *lrn-3(mm200)* (UT1300), *lrn-3(mm200)(UT1301), lrn-3(mm200); mIs12(II)[myo-2p::GFP + pes-10p::GFP + F22B7.9p::GFP]* (UT1302), *lrn-3(mm200) (UT1305).*

Mutagenesis

N2 mid-L4 hermaphrodites were mutagenized by treatment with ethylmethane sulfonate (EMS) essentially as described by Brenner (1974). Briefly, worms were rinsed three times with M9 in a 15 mL conical tube, after which EMS was added to a final concentration of 50 mM. Worms were left on a rocker at 20 for 4 h, after which the worms were rinse with M9 five times to remove the EMS, with waste M9 being disposed of according to institutional chemical waste requirements.

Selection Procedure

Following mutagenesis, worms were grown at 20 °C until eggs were visible on the cultivation plates. The worms were then hypochlorite treated to remove adults (Stiernagle, 2006), and the resultant F1 eggs allowed to develop to L1 arrest overnight, after which they were plated on five fresh NGM/OP50 plates. Following this point, each F1 population of worms was kept separated to maximize the probability.
of recovering independent alleles from each of them. These worms were then allowed to grow until F2 eggs were visible on the cultivation plates, and again hypochlorite treated and left to develop overnight, before plating the resultant F2 worms on fresh NGM/OP50 cultivation plates.

F2 worms were then allowed to develop for 52 hours at 20 °C (to the young adult stage) before being conditioned to benzaldehyde as described below. Worms that chemotaxed toward benzaldehyde in a chemotaxis assay immediately following conditioning were removed from the plate by cutting a 20 mm diameter circle of agar around the benzaldehyde spot and moving it to a fresh NGM/OP50 plate, after which they were left to lay eggs overnight. To protect against accidental loss of a population during the selection process, a piece of agar consisting of approximately 10% of the worms on these plates was then removed and placed on a fresh plate for subsequent cryopreservation. The remaining worms were then hypochlorite treated to remove adults, and the selection process repeated on the worms which developed from the remaining eggs. This selection regimen used in this work was repeated for 4 generations, with each population being maintained separately throughout.

Chemotaxis assays

Chemotaxis assays were performed essentially as per Tsui & van der Kooy (2008). Briefly, worms were washed from NGM growth plates with M9 buffer and allowed to settle to the bottom of a 1.5 mL Eppendorf tube before being placed in the center of a 6 mL 10 cm diameter NGM agar plate lacking a bacterial lawn. Worms were then hand-dried with a Kimwipe until no liquid was visible surrounding them. A 1 uL spot of the test odorant was then placed at one end of the plate, with a 1 uL spot of the solvent it was dissolved in placed at the other as a control. Benzaldehyde was diluted to 1% in ethanol for chemotaxis assays, while isoamyl alcohol and dicaetyl were diluted to 0.1% in ethanol. 1 uL of 1 M NaN₃ was then placed on top of the odorant spot to immobilize the worms, preserving their first odorant choice and eliminating confounding effects of subsequent habituation to the odorant at the test point (Sulston J. E., 1988). Worms were allowed to move freely on the test plates for 1 hour. All chemotaxis experiments were performed at 20 °C.
Approximately 100-300 animals were used per chemotaxis plate, and all results reported here represent the mean of at least 3 independent plates. Chemotaxis indices were normally calculated as the number of animals within a 20 mm radius of the test odorant spot, minus the number of animals within a 20 mm radius of the control odorant spot, divided by the total number of animals on the plate. During the iterative selection procedure, a simplified chemotaxis index calculation was used, wherein animals outside of the two odorant scoring zones were not included in the divisor.

For conditions in which worms were pre-exposed to an odorant for conditioning before the chemotaxis assay, a 2 uL spot of the pure odorant was placed on a piece of parafilm affixed to the lid of the plate, and worms were placed and dried on the agar as described above. In control conditions without an odorant, a similarly sized piece of parafilm was affixed to this lid without the addition of an odorant. As in chemotaxis assays, no bacteria was present on the agar. The plate was then sealed with parafilm and left inverted for the duration of the conditioning period. All conditioning periods used in the work described here were 1 hour in duration.

Pathogenic bacteria learning assays

Pathogenic bacteria learning assays were performed essentially as described by Zhang, Lu, & Bargmann (2005). Briefly, NGM agar plates were inoculated with two non-overlapping zones of bacteria of approximately equal area, consisting of one zone of Pseudomonas aeruginosa PA14 and a second zone of Escherichia coli OP50, and incubated at 37 °C for 24 hours. Young adult (52 h) worms were washed from NGM/OP50 cultivation plates, allowed to settle in 15 mL conical tubes and removed by Pasteur pipette to be placed on the Pseudomonas aeruginosa PA14 zone. Worms were allowed to move freely between the two bacterial zones for 10-14 hours, after which the percentage of worms remaining on the PA14 zone was determined.
**Reagents**

All reagents used in this work except ethanol were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) and stored according to manufacturer’s directions. Ethanol used to dilute odorants for chemotaxis assays was anhydrous Ethyl Alcohol purchased from Commercial Alcohols (Tiverton, ON, Canada).

**Oligonucleotides**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>ins1-internal-f</td>
<td>5’-GATTGAGCTCCACACAGCCA-3’</td>
</tr>
<tr>
<td>ins1-internal-r</td>
<td>5’-CGCGAGTTGTTGGTGCATAG-3’</td>
</tr>
</tbody>
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**Statistical analysis**

Statistical analysis performed using R version 3.0.0. Analysis of Variance calculations performed using the aov function and t tests with the t.test function. Student’s t-tests calculated using 2-sided Welch’s t tests with unequal variance, adjusted afterward for Bonferroni correction, except for comparisons between naïve and conditioned animals, which were calculated using 1-sided Welch’s t tests with unequal variance, adjusted afterward for Bonferroni correction.

Error bars in figures represent the standard error of the means. For figures depicting chemotaxis assay results, the number of test plates used for each condition is indicated for each genotype at the bottom of the figure, except in Figure 9, where the number of test plates used is indicated for each condition.
Results

Iterative selection is a rapid and effective method to isolate *C. elegans* olfactory learning mutants

To quickly isolate new learning mutants, a selective procedure was developed wherein randomly mutagenized, mixed, age-synchronized worm populations were pre-exposed to 100% benzaldehyde and subsequently tested *en masse* for approach to 1% benzaldehyde. The fraction of the worm population which continued to approach benzaldehyde after pre-exposure was then moved to a plate and allowed to reproduce before this selective regimen was reapplied. Although any one benzaldehyde chemotaxis assay had a small error rate after training (insofar as some small fraction of trained worms fail by chance to subsequently avoid benzaldehyde), repeated iterative selection enriched for that portion of the population that consistently went toward benzaldehyde after training, resulting in progressive enrichment for any learning mutants which were present in the mixed population.

In two of the five populations of mutagenized worms which were established, no enrichment (measured by progressively increasing chemotaxis index) was observed across generations, suggesting that no learning mutants had been present in these populations, that mutants which were present were lost because they, by chance, underwent chemotaxis away from benzaldehyde in an early generation, or that these mutants suffered some unrelated selective disadvantage (such as a decreased egg-laying rate) which was a stronger selective force than the chemotaxis assay. Each of the three remaining populations showed a progressive increase in chemotactic index across generations. Population 5 went through a bottleneck in generation 2 in which only a single worm underwent chemotaxis toward benzaldehyde (Figure 1). A subsequent strain derived from the selfed progeny of this worm was named UT1300 and selected for further analysis, while the remaining two populations enriched for trained chemotaxis to benzaldehyde were frozen for future analysis.
Figure 1. Iterative selection of worms for animals which underwent chemotaxis toward benzaldehyde after previous paired exposure to benzaldehyde and starvation resulted in the progressive enrichment of three populations for learning mutants. Populations 1, 4 and 5 all showed progressive increases in positive post-training benzaldehyde chemotaxis, with population 5 undergoing a bottleneck event in generation 2 in which only a single worm underwent chemotaxis to benzaldehyde after training. A strain derived from the progeny of this worm was selected for further analysis.
UT1300 exhibits a pathogenic bacteria learning deficit

To determine the breadth of the learning deficit observed in UT1300, it was tested along with ins-1(nr2091) and lrn-2(mm99) to evaluate deficits in learned aversion to the pathogenic bacteria Pseudomonas aeruginosa PA14. lrn-2(mm99) has previously been shown to exhibit a severe defect in PA14 learning (Wolfe, Merritt, Tong, Stegeman, & van der Kooy, 2015), while ins-1 mutants show severe benzaldehyde learning deficits (C. H. A. Lin et al., 2010). ins-1(nr2091) was found to be wild type for PA14 learning (P>0.10), matching previous reports that ins-6 and ins-7 are the only relevant insulin-like peptides involved in this modality of learning (Chen et al., 2013). UT1300, conversely, was found to have a severe deficit in pathogenic bacteria learning comparable to that found in lrn-2(mm99) strains (Figure 2).

UT1300 is distinct from ins-1(nr2091)

To provide further evidence that the observed phenotype was not the result of accidental contamination with ins-1(nr2091), in frequent use as a positive control in odor/starvation associative learning assays, genomic DNA from ins-1(nr2091), UT1300 and N2 worms were lysed and used as PCR templates with the primers ins1-internal-f and ins1-internal-r, which bind internal to the 2nd and 3rd exons of ins-1 which are absent in ins-1(nr2091). As expected, no amplicon was generated from ins-1(nr2091) template DNA, while a 228bp amplicon was produced using both UT1300 and N2 template DNA.

UT1301 is homozygous at the locus or loci causing the benzaldehyde/starvation learning deficit phenotype

To generate a worm strain homozygous for the mutation or mutations causing the benzaldehyde/starvation learning deficit phenotype, a single UT1300 worm was allowed to self, and 12 F1 worms moved to separate plates before reaching adulthood to establish new populations. All derived populations showed the benzaldehyde/starvation learning phenotype, suggesting that the P0 worm was homozygous at the causative locus or loci (Figure 3). Since this only demonstrated the homozygousity of
Figure 2. UT1300 shows a deficit in olfactory/pathogenic bacteria learning comparable to that of *lrn-2(mm99)*, while *ins-1(nr2091)* is comparable to the wild type (N2) strain. An asterisk indicates significant difference between genotypes (p<0.05 by Bonferroni t test) whereas N.S. indicates not significant. Comparison of strains for altered PA14 pathogenicity learning. A one-way Analysis of Variance revealed a significant effect of strain on CI F(3,16)=14.303 p < 0.0001. A Bonferroni test indicated a significant difference between N2 and UT1300 (p < 0.05), and no significant difference between UT1300 and *lrn-2(mm99)* n.s.
Figure 3. UT1301 is homozygous at the locus or loci causing a benzaldehyde / starvation associative learning deficit. n >=2 for all strains. Of 12 strains derived from individual progeny of a single UT1300 worm, all showed a benzaldehyde/starvation associative learning deficit. A two-way Analysis of Variance between derived strains revealed no significant effect of strain on CI F(11,44)=1.142 n.s. and no interaction between strain and condition F=(11,44)=1.807 n.s. but a significant effect of condition on CI F(1,44)=72.457 p < 0.001.
the particular P0 worm tested (and therefore its selfed progeny), one of the F1-derived populations (UT1301) was chosen for further analysis as a known homozygote.

**UT1301 has a severe deficit in benzaldehyde/starvation associative memory**

To better quantify the severity of the benzaldehyde/starvation learning deficit phenotype of UT1301, the strain was retested as a clonal population in a benzaldehyde/starvation associative learning assay. This revealed that UT1301 had only 22% (calculated as the difference between naïve chemotaxis and trained chemotaxis) of the benzaldehyde/starvation induced inversion of chemotactic behavior as the wild type N2 strain (p < 0.01) (Figure 4). A slight, but not statistically significant (p > 0.10) decrease in naïve chemotaxis was also observed.

**UT1301’s benzaldehyde learning deficit phenotype is likely caused by a recessive mutation at a single gene locus, lrn-3**

To determine the genetic nature of UT1301’s phenotype, it was crossed to a worm carrying an integrated GFP marker on chromosome II (PD4790), enabling easy distinction of cross-progeny from selfed progeny in subsequent crosses. A single F1 worm generated by this cross was then selfed, and the strains originating from single F2s tested for benzaldehyde learning deficits. One (UT1302) in five strains was found to exhibit the phenotype, suggesting that the benzaldehyde learning deficit is caused by a recessive mutation *mm99* at a single gene locus, *lrn-3*.

**UT1301 has a naïve isoamyl alcohol approach deficit phenotype caused by an unrelated mutation to that causing its benzaldehyde learning deficit phenotype**

To further characterize the severity of the learning phenotype in UT1301, it was evaluated for deficits in ability to undergo isoamyl alcohol adaptation. In wild type worms, exposure to isoamyl alcohol, in the presence or absence of food, results in a time-dependent decrease in subsequent approach to isoamyl alcohol (Colbert & Bargmann, 1995). Isoamyl alcohol has been shown to cross-adapt with
Figure 4. UT1301 exhibits a severe deficit in benzaldehyde/starvation associative learning.

Comparison of naïve and trained chemotaxis indices for N2 and UT1301. An asterisk indicates significant difference between genotypes within condition (p<0.05 by Bonferroni t test) whereas N.S. indicates not significant. N2 and UT1301 worms were tested for chemotaxis to benzaldehyde after 1h of starvation alone and 1h of starvation in the presence of benzaldehyde. A two-way Analysis Of Variance revealed a significant interaction between condition and strain F(1,8) = 200.7, p < 0.001. Bonferroni t test between the N2 trained condition and the UT1301 trained condition revealed a significant difference between strains; p < 0.001, but no significant difference between the N2 and naïve conditions n.s.
benzaldehyde, such that worms exposed to benzaldehyde or isoamyl alcohol show corresponding subsequent decreases in approach to the other odorant.

Surprisingly, UT1301 was found to have a severe deficit in naïve approach to isoamyl alcohol (Figure 5a). This deficit in naïve approach was sufficiently severe as to impair our ability to determine whether the strain had a learning deficit. However, the outcrossed strain UT1302 strain showed wild type naïve chemoattraction, revealing a severe isoamyl alcohol learning deficit (Figure 5b).

**Olfactory learning deficits in UT1302 co-occur with learning deficits in some, but not all, odorants.**

Having identified deficits in benzaldehyde learning, isoamyl alcohol learning and pathogenic bacteria learning, we wondered whether the homozygous lrn-3(mm200) allele might impair the ability to pair unconditioned simuli with all odorants. Since both isoamyl alcohol and benzaldehyde are sensed by the paired AWC neurons, and AWC neurons are also partially responsible for sensation of *Pseudomonas aeruginosa* PA14 (Ha et al., 2010), we evaluated the ability of the worm to learn about the odorant diacetyl (butane-2,3-dione), sensed (at low doses) exclusively by the paired AWA neurons (C. I. Bargmann et al., 1993). In contrast with previous forms of olfactory learning we examined in this mutant, UT1301 worms were statistically indistinguishable from wild type worms for diacetyl learning (Figure 6).
Figure 5. UT1301 exhibits a severe isoamyl alcohol chemotaxis deficit, which is genetically dissociable from an isoamyl alcohol learning deficit. For each pane, an asterisk indicates significant difference (p<0.05 by Bonferroni t test) whereas N.S. indicates not significant. A) Comparison between UT1301 and N2 worms for approach to isoamyl alcohol after 1h starvation (naïve) and 1h starvation and isoamyl alcohol (trained). A Bonferroni t test revealed no significant different between naïve and trained UT1301 isoamyl alcohol chemotaxis n.s., while in the N2 control p < 0.01. B) In the outcrossed strain UT1302, a Bonferroni t test revealed no significant difference in chemotaxis to isoamyl alcohol from N2 n.s., but a significant difference between naïve and trained chemotaxis p < 0.05.
Figure 6. UT1302 exhibits wild type naïve and trained diacetyl chemotaxis. An asterisk indicates significant difference between genotypes (p<0.05 by Bonferroni t test) whereas N.S. indicates not significant. Comparison between N2 and UT1302 for approach to diacetyl after one hour’s starvation (naïve) and one hour’s starvation paired with diacetyl exposure (trained). A two-way Analysis Of Variance revealed a significant effect of condition on CI F(1,8)=201.288 p<0.0001, but no effect of strain on CI F(1,8)=2.446 n.s. and no interaction between condition and strain F(1,8)=0.002 n.s. A Bonferroni t test revealed no significant difference between N2 and arr-1(ok401) naïve conditions n.s. and no significant difference between N2 and arr-1(ok401) trained conditions n.s.
Discussion

Selection offers a rapid way to identify learning mutants

Previously identified learning mutants in *C. elegans* have been revealed through the application of screens to large clonal populations of worms (Colbert & Bargmann, 1995; Ishihara et al., 2002; L’Etoile et al., 2002; O’Halloran et al., 2012; Wen et al., 1997) or by candidate gene approaches (Hadziselimovic et al., 2014; Tomioka et al., 2006). While these techniques have succeeded in identifying a variety of genes required for learning, our experience with the first approach has been that it is laborious and identifies mutants very slowly, while the second approach is limited in its ability to identify genes not previously associated with learning pathways. Previous researchers have applied iterative selection to find olfactory mutants in *C. elegans* (C. I. Bargmann et al., 1993), and similar techniques involving selection on mixed populations to identify abnormal phototaxis mutants in *Drosophila* (Benzer, 1967), but to our knowledge the technique has not previously been applied to identify learning mutants. Using this technique, we were able to identify a learning mutant within three weeks.

It is likely that independent mutations causing a learning deficit phenotype exist in populations 1 and 4 (Figure 1), since these populations also showed increased chemotaxis to benzaldehyde after pre-exposure to the odor and starvation relative to N2, and since they were separated from population 7 in the F1 generation. Continued selection on these strains would likely result in a clonal population consisting only of learning mutant worms, showing a trained CI similar to population 7, but the possibility that these populations were already largely clonal, and the learning deficit phenotype was weaker than in population 7, cannot be excluded based on available data. Continued selection on these populations would clarify between these possibilities by revealing whether the CI continued to increase with further iterations of selection (suggesting that the population was not clonal), or remained constant (suggesting that it was, or consisted of a mixed group of mutants each having similar CIs).
Assuming that populations 1 and 4 also contained worms with mutations causing benzaldehyde/starvation learning deficits, this suggests a minimum recovery rate for this phenotype of approximately 1 in 1,000 mutagenized genomes. Since any one population ultimately yielding a learning mutant could have originally contained multiple independent learning mutants, a maximum recovery rate is not possible to calculate based on the data we’ve collected, but this minimum rate suggests (consistent with previous reports) that mutations in diverse loci can result in learning deficit phenotypes.
Arrestin-mediated receptor desensitization is crucial for olfactory discrimination in AWC neurons

Introduction

The ability of worms to find a point of one odorant in a sea of a second odorant sensed by the same neuron, and dependent on the same ODR-3 secondary messenger (C. I. Bargmann et al., 1993; L’Etoile & Bargmann, 2000), is surprising and unintuitive. How can the downstream components of olfactory signaling distinguish ODR-3 released by one receptor from ODR-3 released by another? One possibility is that the point odorant simply contains more total odorant than the surrounding sea (because both the point and sea odorants are present at that spot), and that the apparent ability of worms to find the point odorant is based not on the identity of the odorant but rather on total odorant concentration, but this seems inconsistent with the inability of worms to find a concentrated point of an odorant in a sea of the same odorant.

The sole member of the arrestin family in *C. elegans, arr-1*, is known to play a significant role in olfactory receptor regulation (Palmitessa et al., 2005). We wondered whether the ability of worms to find a point of an AWC- and *odr-3*-dependent odorant, even when surrounded by a sea of a second AWC-, *odr-3*-dependent odorant, might be mediated by the surrounding odorant causing arrestin-mediated desensitization of its corresponding receptor. This desensitization would result in the only active receptor remaining being that corresponding to the point odorant, converting the qualitative difference between the point and sea odorants into a quantitative difference in within-neuron signaling, allowing the worm to find the point odorant without any genuine difference in the identity of the signal transduced by the point and the sea.
Materials and Methods

Nematode propagation

C. elegans were grown in petri dishes on Nematode Growth Media (NGM) and fed OP50 strain Escherichia coli (Wood, 1988). All experiments used well fed young adults (52h) cultivated at 20°C. When necessary, animals were synchronized by hypochlorite treatment according to standard methods (Stiernagle, 2006). Nematode strains were obtained from the Caenorhabditis Genetics Center at the University of Minnesota (St. Paul).

Nematode strains

C. elegans strains used in this work were: wild type C. elegans var Bristol (N2) (Brenner, 1974), arr-1(ok401) (RB660) (Palmitessa et al., 2005) and gpa-2(pk16) gpa-3(pk35) gpa-13(pk1270) V; gpa-5(pk376) gpa-6(pk480) X (GJ0007) (Lans, Rademakers, & Jansen, 2004).

Saturation assays

Saturation assays were performed essentially as described in (C. I. Bargmann et al., 1993). Briefly, worms were washed from NGM growth plates with M9 buffer and allowed to settle to the bottom of a 1.5 mL Eppendorf tube before being placed in the center of a 10 cm diameter petri plate containing 10 mL of NGM agar, to which had been added the saturating odorant (the “sea”). All saturation assays were performed on plates lacking bacterial lawns. Worms were then hand-dried with a Kimwipe until no liquid was visible surrounding them. A 1 uL spot of the test odorant dissolved in ethanol (the “point”) was then placed at one end of the plate, with a 1 uL spot of ethanol placed at the other as a control. 1 uL of 1 M NaN₃ was then placed on top of the odorant spot to immobilize the worms, preserving their first odorant choice. Worms were allowed to move freely on the test plates for 1 hour. All chemotaxis experiments were performed at 20 °C.

Approximately 100-300 animals were used per chemotaxis plate, and all results reported here represent the mean of at least 3 independent plates. Chemotaxis indices were normally calculated as the
number of animals within a 20 mm radius of the test odorant spot, minus the number of animals within a
20 mm radius of the control odorant spot, divided by the total number of animals on the plate.

Reagents

All reagents used in this work except ethanol were purchased from Sigma-Aldrich Inc. (St. Louis,
MO, USA) and stored according to manufacturer’s directions. Ethanol used to dilute odorants for
chemotaxis assays was anhydrous Ethyl Alcohol purchased from Commercial Alcohols (Tiverton, ON,
Canada).

Statistical analysis

Statistical analysis performed using R version 3.0.0. Analysis of Variance calculations performed
using the aov function, and t tests with the t.test function. Student’s t-tests calculated using 2-sided
Welch’s t tests with unequal variance, adjusted afterward for Bonferroni correction.

Error bars in figures represent the standard error of the mean. For figures depicting chemotaxis
assay results, the number of test plates used for each condition is indicated for each genotype at the
bottom of the figure, except in Figure 9, where the number of test plates used is indicated for each
condition.
Results

Olfactory descrimination of isoamyl alcohol and benzaldehyde is arrestin dependent

To test the hypothesis that inter-neuron olfactory discrimination in worms is mediated by arrestin desensitization, we utilized animals homozygous for *arr-1(ok401)*, which contains a deletion in the 3rd, 4th, 5th and 6th (of 10) exons of *arr-1*. This allele eliminates both protein product detectable by western blots (using a polyclonal antibody to ARR-1) and mRNA product detectable by northern blot (using full-length *arr-1* cDNA) and is therefore likely to be null for ARR-1 activity (Palmitessa et al., 2005).

We initially tested the animals alongside the N2 wild type control under identical conditions to those utilized by Bargmann et al., (1993) using a 1uL per 10mL agar sea of benzaldehyde and a 1 uL point of 10% isoamyl alcohol, and found that while N2 worms were able to find the isoamyl alcohol point to a degree comparable to that originally reported, the *arr-1(ok401)* worms were not (Figure 7a). This is unlikely to be due to an uncoordinated phenotype or decreased attractiveness of isoamyl alcohol in this strain because in the absence of a sea odorant the *arr-1(ok401)* strain found the isoamyl alcohol point comparably to N2 (Figure 7b).

Saturation of benzaldehyde response is partially arrestin dependent

The concentration of the odorant “sea” used above was originally chosen because it resulted in abrogated chemotaxis to a 1uL point of 0.5% benzaldehyde, which is otherwise (in the absence of the sea odorant) sufficient to drive strong chemotaxis (C. I. Bargmann et al., 1993), suggesting that response to this odor had been saturated. We next wondered whether the abrogated benzaldehyde response seen in the presence of the sea odorant was not due to saturation of the response, but rather due to arrestin-mediated down-regulation of the benzaldehyde receptor or receptors. To test this possibility, we evaluated chemotaxis to 1uL points of a range of concentrations of benzdehyde in a 1uL per 10mL agar benzaldehyde sea, in both *arr-1(ok401)* and N2 worms. While N2 worms were found to fail to undergo chemotaxis to the point source, matching the previous reports, *arr-1(ok401)* worms were seen to have a
Figure 7. The arr-1(ok401) mutation selectively eliminates the ability of worms to find a point of isoamyl alcohol in a sea of benzaldehyde. An asterisk indicates significant difference between genotypes within condition (p<0.05 by Bonferroni t test) whereas N.S. indicates not significant. **A)** Comparison between N2 and arr-1(ok401) for approach to a 10% spot of isoamyl alcohol in a sea of 0.01% benzaldehyde. A Bonferroni t test revealed a significant difference in CI; p<0.05. **B)** Comparison between N2 and arr-1(ok401) for approach to a 10% spot of isoamyl alcohol in the absence of a second odorant. A Bonferroni t test revealed no significant difference in CI n.s.
partially restored ability to find the point source of benzaldehyde across a range of concentrations (Figure 8).

**AWA-sensed odor discrimination is also arrestin-dependent**

To determine whether all within-neuron discrimination of odorants might be dependent on arrestin-mediated desensitization, we next evaluated the ability of N2 and *arr-1(ok401)* worms to find a point source of the AWA-sensed odorant diacetyl in a sea of the AWA-sensed odorant pyrazine. While N2 worms underwent robust chemotaxis to points of both 0.1% and 1% diacetyl in a sea of 1% pyrazine, *arr-1(ok401)* worms exhibited a reduced ability to find the diacetyl point (Figure 9a). Similarly, when a 0.01% concentration of diacetyl was used as the sea odorant and varying concentrations of pyrazine as the point odorant, reduced chemoattraction to the pyrazine point was seen across all concentrations (Figure 9b).

In both the diacetyl point in a pyrazine sea and the pyrazine point in a diacetyl sea paradigms, the effect of *arr-1* loss of function was stronger at low concentrations of the point odorant than at high concentrations. This is consistent with within-neuron olfactory discrimination being ARR-1-dependent, since at sufficiently high concentrations the point odorant will be detectable simply because it results in greater odr-3 release than the surrounding sea, but may also be attributable to weak point odorant activation of an additional low-affinity receptor or receptors in other neurons at high concentrations.

**Benzaldehyde/starvation olfactory learning is independent of non-odr-3 alpha subunits**

Although *odr-3* is the primary alpha subunit involved in olfactory signal transduction in AWC, five other alpha subunits (*gpa-2, gpa-3, gpa-5, gpa-6, gpa-13*) are known to be expressed in this cell (C. I. Bargmann et al., 1993; Lans et al., 2004; Roayaie et al., 1998). Although individual loss of function mutations in these other alpha subunits show no olfactory phenotype in the wild type background, the same mutations in a *odr-3* knockout background reveal moderate stimulatory or inhibitor roles for these genes in G-protein signaling (Lans et al., 2004). Our finding that olfactory discrimination in AWC
Figure 8. *arr-1(ok401)* displays a trend whereby ability to find a point of benzaldehyde in a sea of benzaldehyde is partially restored. Chemotaxis to 0.1%, 0.5% and 1% points of benzaldehyde in a sea of 0.01% benzaldehyde. A two-way Analysis of Variance revealed a significant effect of strain on CI $\text{F}(1,50)=4.419 \ p<0.05$ but no effect of Concentration on CI $\text{F}(2,50)=2.485 \ n.s.$ and no interaction between strain and concentration $\text{F}(2,50)=2.053 \ n.s.$
Figure 9. AWA-sensed odor discrimination is arr-1 dependent.  
A) Chemotaxis to a point of 0.1% and 1% diacetyl in a sea of 1% pyrazine. A two-way Analysis of Variance revealed a significant effect of strain on CI $F(1,26)=5.182 \ p<0.05$, but no effect of diacetyl concentration $F=\text{n.s.}$ or interaction between condition and strain $F=\text{n.s.}$  
B) Chemotaxis to a point of 0.001%, 0.01%, 0.1% and 1% pyrazine in a sea of 0.01% diacetyl. A two-way Analysis of Variance revealed a significant effect of strain on CI $F(1,50)=11.440 \ p<0.01$ and a significant effect of pyrazine concentration on CI $F(1,50)=38.1 \ p<0.0001$, but no interaction between strain and pyrazine concentration $\text{n.s.}$
depended on arrestins raised the question of whether the five non-\textit{odr}-3 alpha subunits known to be expressed in AWC might play a role unrelated to olfactory discrimination.

The fact that some of these alpha subunits were stimulatory and some inhibitory suggested to us that they might be involved in mediating experience-dependent plasticity in chemotactic response to odorants (Lans et al., 2004). To evaluate this possibility, we tested GJ0007, a strain in which all alpha subunits known to be expressed in AWC except \textit{odr}-3 are knocked out, for defects in benzaldehyde/starvation associative learning. GJ0007 showed a mild but statistically significant increase in naïve benzaldehyde approach, and a corresponding mild but significant decrease in trained benzaldehyde aversion (Figure 10). Although the latter finding considered in isolation may suggest a hindered ability to engage in olfactory plasticity, when considered in the context of increased naïve chemotaxis it is most consistent with wild type benzaldehyde learning with strengthened attraction.
Figure 10. Benzaldehyde/starvation olfactory learning depends on non-ODR-3 alpha subunits. An asterisk indicates significant difference between genotypes within condition (p<0.05 by Bonferroni t test) whereas N.S. indicates not significant. Comparison between N2 and GJ0007 (gpa-2(pk16) gpa-3(pk35) gpa-13(pk1270) gpa-5(pk376) gpa-6(pk480) for approach to benzaldehyde after one hour’s starvation (naïve) and one hour’s starvation paired with benzaldehyde exposure (trained). A two-way Analysis of Variance between N2 and GJ0007 revealed a main effect of strain on CI F(1,39)=21.636 p < 0.001, and a main effect of condition on CI F(1,39)=102.331 p < 0.001, but no interaction between strain and condition F(1,39)=1.168 n.s. A Bonferroni t test revealed a significant difference in both naïve and trained CI between GJ0007 and N2 p<0.05.
Discussion

Olfactory discrimination within AWC likely occurs via arrestin-mediated desensitization

Our finding that loss of \textit{arr-1(ok401)} selectively interferes with the ability of \textit{C. elegans} to locate a point of one AWC-sensed odorant in a sea of another, in conjunction with our finding that loss of \textit{arr-1} has no corresponding effect on chemotaxis to points of odorants alone, suggests a model for olfactory discrimination within AWC, and a reinterpretation of previously reported results.

Previous investigations of worm olfaction have suggested that worms were capable of distinguishing between odors sensed within single neurons (C. I. Bargmann et al., 1993; Roayaie et al., 1998). In our model, worms are not capable of distinguishing between alpha subunits released by one olfactory receptor within AWC, and those released by another. Rather, the ability of worms to find points of odorants in seas of another odorant is attributable to desensitization of the receptors responsible for sensing the sea odorant, such that the receptors corresponding to the point odorant are far more efficacious in causing alpha subunit release. Although signal transduction of sensation of the point and the sea are indistinguishable downstream of the receptor, both converging on alpha subunit release in the same cells, arrestin-mediated desensitization of very active sea odorant receptors allows the worm to chemotax to the point accurately.

At the molecular level, our model suggests that when \textit{C. elegans} is surrounded by a high concentration of an AWC-sensed odorant, a ligand-induced conformational change leading to subsequent G-protein release in the corresponding receptor or receptors occurs. This conformational change allows for phosphorylation of these receptors, perhaps by GRK-1 or GRK-2 (although \textit{grk-2} is not known to be expressed in AWC (McKay et al., 2003), or possibly by another kinase. Phosphorylation of the receptor allows for binding of ARR-1, resulting in desensitization of the receptor. This desensitization decreases the G-protein alpha subunit release caused by activation of the receptor corresponding to the sea odorant.
Since ligand-binding of the receptor or receptors responsible for sensation of the point odorant occurs less frequently than for the sea odorant (since there is a lower density of ligand present), it is unaffected or less affected by arrested mediated desensitization, and continues releasing G protein alpha subunits in response to ligand presence. The rate of alpha subunit release, then, is primarily affected by proximity to the point odorant, allowing for chemotaxis despite the presence of the surrounding sea.

Previous researchers have interpreted the ability of worms to find a point of one odorant within a sea of a second odorant sensed by the same neuron to be evidence for discrimination between these odorants (C. I. Bargmann et al., 1993). In our model, rather than being indicative a genuine ability to discriminatory between these odorants, the ability to locate the point odorant within the sea odorant is caused by the selective desensitization of the receptor or receptors corresponding to the sea odorant, without any discrimination occurring downstream of the olfactory receptors.

**Olfactory discrimination in AWA is partly dependent on arrestin-mediated desensitization**

Comparable to our findings for AWC-sensed odorants, discrimination between the AWA-sensed odorants pyrazine and diacetyl was found to be partially *arr-1* dependent across a range of concentrations. Unlike odorants sensed by AWC, however, the *arr-1(ok401)* mutation did not fully eliminate chemotaxis to the points of either AWA-sensed odorant. Consistent with this finding, previous researchers have reported that loss of function mutations in *arr-1* result in less severe deficits in olfactory adaptation for AWA-sensed odorants than for AWC-sensed odorants (Palmitessa et al., 2005), possibly supporting an *ARR-1* independent mechanism of receptor desensitization in AWA.

Alternatively, both diacetyl (Taniguchi, Uozumi, Kiriyama, Kamizaki, & Hirotsu, 2014) and pyrazine (Sengupta, Colbert, & Bargmann, 1994) are known to be weakly sensed by non-AWA neurons. Differential activation of these weakly sensing olfactory neurons may provide worms with sufficient discriminatory ability to locate the point within the sea partially independent of arrestin desensitization of receptors.
Chapter 4 General Discussion

The iterative screen employed in this work rapidly identified a benzaldehyde/starvation associative learning mutant, supporting the hypothesis that this technique allows for far more rapid identification of mutants than traditional screens of clonal populations. Although the genetic identity of *lrn-3(mm200)* remains unknown, it has a broad suite of olfactory learning deficits and a relatively penetrant phenotype, making it a promising mutation for subsequent analysis.

The hypothesis that arrestin desensitization of olfactory receptors plays a significant role in the ability of worms to distinguish between two odors sensed by the same neuron was also supported. Olfactory discrimination within AWA and AWC were found to be largely depending on arrestin desensitization. The effect of sea “saturation” of odorant responses reported by Bargmann et al. (1993) was also found to be arrestin-dependent, with the additional signaling possible in the absence of ARR-1 revealing a partially restored ability to find a point of an odorant in a sea of the same odorant.

**Olfactory responses in *C. elegans* are mediated by dissociable discrimination and learning components.**

Previous work has demonstrated that non-associative and associative olfactory learning modalities in *C. elegans* could be genetically dissociated using *arr-1* and *ins-1* mutations, such that non-associative learning was dependent on *arr-1* and associative learning on *ins-1* (Pereira & van der Kooy, 2012). The results presented here further show that the initial processes of olfactory discrimination between odorants sensed by the same neuron are dependent on ARR-1. In combination with the finding of Pereira & van der Kooy that *arr-1* loss of function mutations do not result in benzaldehyde learning deficits, these findings suggest a genetic dissociation between the processes of initial sensory discrimination and subsequent associative learning.

Our findings also suggest, however, that non-associative learning (or ARR-1-mediated sensory adaptation) is required for successful discrimination of within-neuron sensed odors in *C. elegans*. This
entanglement of non-associative learning and sensory discrimination is likely avoided within olfactory sensory neurons in mammals by the presence of only one or few olfactory receptors per neuron, although a similar entanglement in downstream neurons remains possible (and indeed, is known to occur in other sensory modalities (Defrin, Pope, & Davis, 2008)).

Although previous studies have shown that arrestin-mediated downregulation is required for climbing of chemosensory gradients by human leukocytes (Aragay et al., 1998), ours is the first to reveal a requirement for arrestin-mediated receptor downregulation for sensory discrimination in a chemosensory gradient task.

**Arrestin desensitization acts to increase or decrease the contrast between point and sea depending on paradigm**

While the *arr-1(ok401)* mutation prevented worms in the different odorant (isoamyl alcohol in a sea of benzaldehyde) paradigm from locating the point odorant (Figure 7), it partially restored the ability of worms in the same odorant (benzaldehyde in a sea of benzaldehyde) paradigm to find the point (Figure 8). This unintuitive finding is best understood by consideration of the effect of *arr-1* loss of function on the contrast between the point odorant and the sea odorant.

In the different odorant paradigm, arrestin acts to reduce signaling caused by the sea odorant by desensitizing the corresponding receptor or receptors. Since the worms are exposed to far fewer molecules of the point odorant, its receptor or receptors are far less frequently activated, and so remain unbound by arrestin. This desensitization of sea odorant sensation, while point odorant sensation remains spared, has the effect of increasing the contrast between signaling caused by the point odorant and that caused by the sea, allowing for accurate chemotaxis to the point. In the absence of arrestin, however, enhanced signaling from the sea odorant makes this contrast far weaker, preventing worms from accurately locating the point.
Conversely, in the same odorant paradigm, arrestin desensitization imposes a ceiling on maximum odorant signaling. Although the point consists of a much higher concentration of benzaldehyde than the surrounding sea, the limitation imposed by this ceiling on receptor activation prevents this contrast from being transduced downstream of the receptor. In the absence of arrestin, this ceiling on receptor activation is lifted, allowing for the point to cause greater receptor activation than the surrounding sea, and for chemotaxis to the point to be partially restored.

The key importance of contrast in performing the point in a sea task is further illustrated by the effect of point concentration on ability to locate a point of diacetyl in a sea of pyrazine, or a point of pyrazine in a sea of diacetyl (Figure 9a and 8b). In both cases, a trend is visible wherein increased concentration of the point reduces the impact that arr-1 loss of function has on the worm’s ability to complete the task by increasing the contrast between the point and surrounding sea.

**Arrestin loss of function reveals a weak aversive component to the point in the sea task**

In the benzaldehyde in a sea of benzaldehyde paradigm, we observed moderate, dose-dependent negative chemotaxis (away from the point) at several concentrations in the N2 strain (Figure 8). This matches data previously reported but not addressed by Bargmann et al. (1993) (Figure 4) that slight negative chemotaxis could be seen in this condition, and further shows that the extent of negative chemotaxis depends on the concentration of the point. Supporting our hypothesis that arrestin desensitization is required for within-neuron olfactory discrimination, the isoamyl alcohol in a sea of benzaldehyde paradigm was correspondingly found to cause a slight negative aversive chemotaxis in the absence of arr-1 (Figure 4a), showing that without arrestin mediated desnsensitization the point of isoamyl alcohol was indistinguishable from a point of benzaldehyde.

Among odorants sensed by AWA, negative chemotaxis can be seen in the arr-1(ok401) mutant in the pyrazine in a sea of diacetyl paradigm (Figure 9b) but not in the diacetyl in a sea of pyrazine paradigm
(Figure 9a), at least at the doses tested. This is partially consistent with the previously reported absence of negative chemotaxis reported for the diacetyl in a sea of diacetyl condition (C. I. Bargmann et al., 1993).

Several possible explanations exist for the negative chemotaxis observed in these conditions. The high concentrations of odorants present at the point may be treated aversively by the animal: in the case of benzaldehyde, aversive odorant sensation is known to occur at high concentrations, and have been localized to different neurons from those mediating attractive olfaction (Colbert, Smith, & Bargmann, 1997). Aversive odorant sensation by other sensory neurons may also occur with high doses of other odorants, having been masked in other paradigms by more significant attractive odorant signaling. Should the attractive signaling provide insufficient contrast to allow for directed chemotaxis, as in some of our experiments using *arr-1* animals, weak aversive signaling could dominate. Alternatively, the extended exposure to the sea odorant in the absence of food could cause weak learned aversion to it, manifested by aversion to the slightly higher concentrations present at the point source of the odorant.

*Arrestin-dependent olfactory discrimination suggests a mechanism for the observed cross-adaptation between odorants*

Previous research has explored the cross-adaptation seen between training to isoamyl alcohol and benzaldehyde in *C. elegans* (Atkinson-Leadbeater, Nuttley, & van der Kooy, 2004; Colbert & Bargmann, 1995). Our finding, that the initial components of olfactory discrimination depend on arrestin-mediated downregulation of olfactory receptors, suggests that isoamyl alcohol and benzaldehyde may cross-adapt because there is no cellular mechanism downstream of the olfactory receptor capable of genuinely distinguishing between the signaling cascades transduced by binding of these two odorants to their corresponding olfactory receptors. Under this interpretation, all odorants sensed exclusively by a fully-overlapping set of neurons would exhibit cross-adaptation.

It is known that cross-adaptation in the “Hawaiian” *C. elegans* strain, CB4856, proceeds only from benzaldehyde to isoamyl alcohol, with neither benzaldehyde adaptation or cross-adaptation from
isoamyl alcohol to benzaldehyde occurring. Our findings suggest that this may be indicative of an alteration of the localization of benzaldehyde or isoamyl alcohol sensation in this strain, such that one of these odorants was sensed in neurons not fully overlapping the set sensing the other. The neurons in CB4856 which were responsible for sensing benzaldehyde, in this interpretation, would not themselves be able to induce a trained state in the animal, but could signal appropriately to drive reduced benzaldehyde chemotaxis in response to isoamyl alcohol training sensed by other neurons.

**Negative associative olfactory learning is not dependent on non-odr-3 alpha subunits**

Our finding that non-odr-3 G-protein alpha subunits are not required for negative associative olfactory learning suggests that the inverted chemotactic valence of benzaldehyde seen in wild type worms after benzaldehyde/starvation training does not depend on altered binding, transcription or recruitment of non-odr-3 alpha subunits.

That both naïve and trained chemotaxis to benzaldehyde is increased in animals in which all non-odr-3 alpha subunits are knocked out suggests that the net effect of these subunits is to have a minor inhibitory effect on AWC-mediated odorant chemotaxis, and that this effect is not dependent on past experience. That the non-odr-3 alpha subunits are not required to mediate learned behavior suggests that in learned, as in naïve chemotactic behavior, they are minor components of signal transduction. These other alpha subunits may play a more substantial role in another, non-chemotactic GPCR pathway, or in a chemotaxis pathway present in another neuron.

**The point in the sea task may offer a facile technique for localizing olfactory sensitivity to particular sensory neurons**

The requirement for arrestin downregulation for accurate discrimination of one odorant from another sensed by the same neuron suggests a technique by which the neuron responsible sensing odorants in *C. elegans* might readily be identified. Should a library of odorants with known, highly specific locations of sensation be produced, it might be used to rapidly screen a candidate odorant having
an unknown location of chemosensation in a point in the sea task. Point odorants sensed exclusively by the same neuron or neurons as a sea library odorant would be expected to be locatable only by N2 worms, while both N2 and arr-1 worms would successfully undergo chemotaxis to library odorants sensed by all other neurons. This technique may provide a facile early screening technique to localization sensation of odorants, reducing the number of time-consuming and error-prone laser ablation experiments required for subsequent confirmation.

**Homozygous mutation at the mm200 locus causes severe, but not complete, benzaldehyde learning deficits.**

Over 75% of the change in benzaldehyde CI after pre-exposure to benzaldehyde and starvation is eliminated in lrn-3(mm200) worms, suggesting that lrn-3 is a key gene involved in the benzaldehyde learning pathway. The small remaining ability of homozygous lrn-3(mm200) animals to learn about benzaldehyde may be caused by the mutation resulting in only a partial loss of function in the protein, or by the mutation occurring in one of two partially redundant pathways, each of which has a partial effect in mediating learning. Loss of function mutations in the gene encoding the insulin-like-protein ins-1 or its sole receptor daf-2 cause a complete failure to learn about many AWC-sensed odors (C. H. A. Lin et al., 2010), in addition to stimuli not sensed by AWC such as salt (Tomioka et al., 2006) and temperature (Kodama et al., 2006), suggesting that if partially redundant pathways exist, they contain at least some overlapping genes. In either case, it is possible that conditions exist (such as pre-exposure times, spaced training, increased or alternate testing durations or odorants) which would reveal a full block of learning in strains carrying a homozygous mm200 mutation.

**lrn-3 may be required for wild type learning to all AWC-sensed odors**

The failure of strains containing lrn-3(mm200) to fully learn about several odorants known to be sensed by AWC, and the ability of animals of these strains to learn about diacetyl, known to be sensed at low concentrations exclusively by AWA (Sengupta et al., 1996), suggests that lrn-3 may play a role in
learning about all AWC-sensed odors. If so, it is likely expressed in AWC, or perhaps in a neuron (such as AIA, AIY or AIB) immediately downstream of AWC (White et al., 1986).
Chapter 5 Conclusions

This work shows that iterative selection to identify learning mutants offers a powerful strategy by which large numbers of these animals might be found for further study and analysis. Through its use, it is possible to exploit the advantages of unbiased forward genetic screens without the considerable labor required to analyze clonal populations.

The findings of this work challenge the traditional view, that worms are able to discriminate between different odorants sensed by a single neuron, by suggesting that the apparent olfactory discrimination results from arrestin-mediated desensitization of the receptor for one of the odorants. These findings suggest that the genuine range of olfactory discrimination in worms is probably constrained to the number of chemosensory neurons available, perhaps slightly expanded for cases in which odorants are sensed combinatorially by multiple neurons.

This work suggests both new similarities and new differences between mammalian and worm olfaction. While the apparent ability of worms to discriminate between several odorants sensed by a single neuron suggested a fundamental difference in olfactory signal transduction between worms and mammals (which express only a single receptor per olfactory neuron, and determine odorant identity by the combinatorial activation of many different receptors in many neurons), our findings suggest that worms, like mammals, are not capable of intra-neuron olfactory discrimination. Conversely, worms having a comparable number of olfactory receptors to mammals, in conjunction with evidence that binding at one olfactory receptor within a neuron could be distinguish from binding at another, suggested that worms may be able to discriminate a similar range of smells to mammals despite their paucity of olfactory neurons. This work suggests that this view is incorrect, and that the range of genuine olfactory discrimination in C. elegans is vastly smaller than the range of mammalian olfactory discrimination.
Finally, this work shows that the slight aversive chemotaxis first observed in the benzaldehyde in a sea of benzaldehyde paradigm by Bargmann et al., (1993) is a dose-dependent phenomenon, and one which is generalized across odorants in the absence of arrestin-mediated discrimination.
Chapter 6 Future Directions

To further characterize the nature of the \textit{lrn-3(mm200)} mutation, the UT1305 strain will be outcrossed to PD4790 (which contains a GFP marker integrated into an N2 background, allowing cross-progeny to be easily distinguished from selfed-progeny) resulting in a total of 3x outcrosses. The resulting strain will then be sequenced using whole genome sequencing, and mutations in candidate loci identified. In cases where other strains harboring mutations in candidate loci exist, they will be tested in the benzaldehyde/starvation associative learning assay for learning deficits, and mutations which recapitulate the learning phenotype observed in \textit{lrn-3(mm200)} identified. The identity of \textit{lrn-3(mm200)} will then be confirmed by rescue with fosmids containing the genomic regions encompassing the identified mutations. Additional alleles of the \textit{lrn-3} gene will be sought through the \textit{Caenorhabditis} Genetics Center and other public repositories. Should no unambiguous null alleles be available to evaluate the null phenotypes, one will be generated using the CRISPR-Cas9 system (Friedland et al., 2013).

Although \textit{lrn-3(mm200)} animals retain positive chemotaxis to benzaldehyde after training, a slight decrease in their chemotactic index is present relative to naïve animals. It is possible that this small retained ability to learn is caused by \textit{lrn-3(mm200)} being a hypomorphic allele, by it affecting a phase of memory only weakly expressed under our training and testing conditions, or by it affecting only one of multiple, partially redundant parallel pathways. To better understand the phase of memory \textit{lrn-3} effects, and to characterize this small retained ability to learn, training and testing conditions will be altered to investigate very short- and very long-term memories. One hour massed training followed by half-hour testing will be evaluated in \textit{lrn-3} to determine whether a full deficit (or the complete absence of a deficit) of short-term learning is present in the animal (Stein & Murphy, 2014), while repeated spaced training followed 16 hours later by 1 hour testing will be evaluated to determine the effect of mutations in the gene on long-term memory. These experiments will be conducted with a null allele of the gene, should one be identified or produced.
After identification and confirmation of the causative locus of *lrn-3(mm200)*, the anatomical location or locations of gene expression will be determined through construction of a *lrn-3p::GFP* transgenic worm. The location or locations of expression required for wild type learning will then be established through cell-specific rescue of *lrn-3*.

Genes interacting with *lrn-3* may then be identifiable using a candidate gene approach, based on known interactors of homologous genes.

To confirm the generality of our findings on within-neuron olfactory discrimination, a dose-response curve for points of isoamyl alcohol in a sea of benzaldehyde will be constructed, with the anticipation that sufficiently high concentrations of isoamyl alcohol may partially restore the ability of worms to complete the point in the sea task by increasing the contrast between signaling caused by the two odorants.

It is possible that the effect of *arr-1* loss of function mutations on performance in the point in the sea task is caused by the disruption of GPCR-regulation during development, rather than by elimination of downregulation of olfactory receptors during sensation. To confirm that this is not the case, *arr-1* will be expressed under a heat-shock promoter in an *arr-1(ok401)* background, and transcription of the gene induced by heat-shock at the adult stage. The animals will subsequently be tested in the point in the sea task to confirm rescue of ARR-1 function.

To investigate the cause of the negative chemotaxis seen under some point in a sea conditions, *ins-1(nr2091)* mutants, defective in most associative learning paradigms, will be tested under conditions generating negative chemotaxis in wild type animals. If the observed negative chemotaxis in these paradigms is due to associative olfactory learning, it is expected that *ins-1* mutant animals will exhibit neutral chemotaxis in them.
To confirm that non-odr-3 alpha subunits are not required for olfactory discrimination, GJ0007, a strain in which all alpha subunits known to be expressed in AWC except odr-3 have been knocked out, will be tested to determine if it is able to find a point of one AWC-sensed odor in a sea of a second.

To investigate the identity of the kinase responsible for phosphorylating odorant receptors prior to arrestin binding, worms will be fed RNAi against grk-1 and grk-2, and subsequently tested in a point in the sea task to determine whether knockdown of these genes results in partial phenocopying of the arr-1(ok401) mutation. To confirm that phosphorylation of olfactory receptors olfactory receptors occurs in response to receptor activation in C. elegans, a phosphoshift assay will be conducted on ODR-10 – the diacetyl receptor – in worms exposed to diacetyl and unexposed control worms.

To determine whether the location of benzaldehyde sensation in the CB4856 “Hawaiian” strain is altered, supporting our explanation for the altered benzaldehyde/isoamyl alcohol adaptation and cross-adaptation observed in this strain, the ceh-36(ky640) allele, encoding a loss of function mutation in the CEH-36 homeodomain transcription factor required for development of the AWC neurons (which sense benzaldehyde in the wildtype strain (Koga & Ohshima, 2004)), will be crossed into CB4856. The resultant strain will then be backcrossed several times to CB4856, retaining progeny containing the ceh-36(ky640) allele, before being selfed to generate animals homozygous for ceh-36(ky640). This strain, containing the ceh-36(ky640) mutation in a CB4856 background, will then be tested to determine whether it exhibits greater chemotaxis toward benzaldehyde than the severely abrogated benzaldehyde chemotaxis observed in animals with the ceh-36(ky640) mutation in an N2 background, suggesting an AWC-independent site of benzaldehyde chemosensation.

The work described here identifies a potentially novel learning mutant, and offers a reinterpretation of the apparently olfactory discriminatory capabilities of C. elegans. Further investigation of these results may shed considerable light on these fundamental processes in C. elegans neurobiology.
References


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