DOPAMINE AND PDF SIGNALING MEDIATE HABITUATION TO REPEATED ACTIVATION OF A POLYMODAL NOCICEPTOR IN *CAENORHABDITIS ELEGANS*

by

EVAN ARDIEL

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Neuroscience)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

April 2015

© Evan Ardiel, 2015
Abstract

Habituation is a highly conserved phenomenon that remains poorly understood at the molecular level. Invertebrate model systems, like *C. elegans*, can be a powerful tool for understanding this fundamental process. To expand our knowledge of habituation I developed a high-throughput learning assay using real-time computer vision software for behavioral tracking and optogenetics for stimulation of a *C. elegans* polymodal nociceptor pair, ASHL and ASHR. These cells are especially interesting in the context of habituation because of the diversity and salience of the stimuli they detect. Photoactivation of ASH promoted backward locomotion and persistent stimulation altered the magnitude of this response in a manner consistent with the key behavioral characteristics of habituation. The decrement in reversal duration was readily reversed by a dishabituating stimulus, in this case non-localized mechanosensory input detected by the touch receptor neurons. In addition to altering the response properties, repeated ASH activation suppressed spontaneous reversals and accelerated forward movement. Food and dopamine signaling (bas-1, cat-4, cat-2, trp-4) promoted responding to persistent ASH activation and I identified the D1-like dopamine receptor, DOP-4, as the key mediator. Neuropeptide synthesis mutants (egl-3 and egl-21) displayed impaired plasticity for a variety of behavioral metrics, prompting me to perform an RNAi screen targeting neuropeptide receptors. From this screen, I implicated pigment dispersing factor (PDF) signaling in habituation of response latency and duration. Failure to avoid some stimuli detected by ASH could be fatal for *C. elegans*, so why
do the reversal responses habituate? My data indicate that habituation is part of a strategy to promote dispersal.
Preface

This dissertation is original unpublished work. I was the lead investigator for the projects discussed in chapters 2 and 3. Giles AC was involved in the early stages of concept formation. Lee A, Huen M, Yu A, Liang A, and McEwan A constructed strains or reagents for the project. Giles AC and Yu A contributed to data collection. Rankin CH was the supervisory author throughout. Portions of the introductory chapter are modified from a review paper on which I am the primary author: Ardiel, E.L., Rankin, C.H. (2010) An elegant mind: learning and memory in Caenorhabditis elegans. Learn Mem, 17(4):191-201.
Table of contents

Abstract ................................................................................................................................. ii
Preface ................................................................................................................................. iv
Table of contents ................................................................................................................ v
List of tables ........................................................................................................................ vii
List of figures ...................................................................................................................... viii
Acknowledgements ........................................................................................................... ix
Dedication ............................................................................................................................. x

1. Introduction ..................................................................................................................... 1
   1.1 Habituation ............................................................................................................... 1
   1.2 C. elegans ............................................................................................................... 5
   1.3 C. elegans habituates ............................................................................................. 7
      1.3.1 Mechanosensory habituation .......................................................................... 7
      1.3.2 Chemosensory habituation ............................................................................ 13
   1.4 ASH: Polymodal nociceptors .................................................................................. 15
      1.4.1 Molecules, circuits, behaviors ......................................................................... 15
      1.4.2 Modulation ....................................................................................................... 23
   1.5 Conclusion ............................................................................................................... 29
   1.6 General methods ..................................................................................................... 30
      1.6.1 Strains .............................................................................................................. 30
      1.6.2 Behavioral tracking ......................................................................................... 31
      1.6.3 Statistics .......................................................................................................... 32

2. Role of food and dopamine in habituation ................................................................. 34
   2.1 Introduction .............................................................................................................. 34
   2.2 Methods .................................................................................................................. 36
      2.2.1 Strains .............................................................................................................. 36
      2.2.2 Behavioral tracking ......................................................................................... 37
      2.2.3 Nose touch ....................................................................................................... 37
      2.2.4 Octanol exposure ............................................................................................ 38
   2.3 Results ..................................................................................................................... 38
      2.3.1 Population assay for repeated ASH activation ................................................. 38
      2.3.2 Generalization of photoactivation and naturally sensed stimuli ...................... 40
      2.3.3 Dishabituation ................................................................................................. 41
      2.3.4 Dopamine signaling slows habituation ............................................................ 43
      2.3.5 DOP-4 signaling slows habituation ................................................................. 46
      2.3.6 Habituation promotes dispersal ....................................................................... 47
   2.4 Discussion ................................................................................................................ 48

3. Role of PDF signaling in habituation ........................................................................... 63
   3.1 Introduction .............................................................................................................. 63
   3.2 Methods .................................................................................................................. 65
      3.2.1 Strains .............................................................................................................. 65
      3.2.2 Plasmid construction ...................................................................................... 66
      3.2.3 RNAi ................................................................................................................. 68
   3.3 Results ..................................................................................................................... 69
3.3.1 glr-1 phenotypes ........................................................................................................ 69
3.3.2 Loss of egl-3 suppresses glr-1 .................................................................................. 70
3.3.3 GPCR RNAi suppressor screen ................................................................................ 72
3.3.4 PDF signaling mediates habituation ....................................................................... 73
3.3.5 PDFR-1 functions in neurons and muscle .................................................................. 75
3.3.6 PDF signaling promotes dispersal to persistent sensory input ............................... 77
3.4 Discussion ................................................................................................................ 79

4. Discussion .................................................................................................................. 100
   4.1 A new high-throughput habituation assay ................................................................. 101
   4.2 Similarities, differences, and interactions of converging circuits ......................... 105
   4.3 Conclusion ............................................................................................................... 109

References ..................................................................................................................... 111
List of tables

Table 3.1 GPCR loss of function phenotypes................................................................. 96
Table 3.2 Expression pattern of pdfr-1 and Cre promoters. .............................................. 98
Table 3.3 PDFR-1 rescue experiment summary................................................................. 99
List of figures

Figure 1.1 Wiring diagram for tap and ASH-mediated reversals............................... 33
Figure 2.1 Plasticity of reversal responses .................................................................. 53
Figure 2.2 Shift in foraging behaviors ...................................................................... 54
Figure 2.3 Spontaneous recovery from training......................................................... 55
Figure 2.4 Generalization of stimuli......................................................................... 56
Figure 2.5 Dishabituation......................................................................................... 57
Figure 2.6 Dopamine signaling promotes responding.............................................. 58
Figure 2.7 Loss of dop-4 recapitulates cat-2 phenotype........................................... 59
Figure 2.8 Locomotory behavior of the dop-4 mutant ............................................. 61
Figure 2.9 Habituation training promotes dispersal............................................... 62
Figure 2.10 Phenotypes for glutamate transmission mutants.................................... 83
Figure 3.2 Suppression of the glr-1 phenotypes....................................................... 85
Figure 3.3 Neuropeptide synthesis mutants............................................................. 86
Figure 3.4 GPCR RNAi screen.............................................................................. 87
Figure 3.5 PDF signaling promotes habituation...................................................... 88
Figure 3.6 pdfr-1 mutant phenotype....................................................................... 89
Figure 3.7 Restoring pdfr-1 expression................................................................. 90
Figure 3.8 PDF signaling promotes dispersal......................................................... 92
Figure 3.9 PDF signaling promotes dispersal during habituation training............... 93
Figure 3.10 Persistent sensory input promotes dispersal ....................................... 95
Acknowledgments

Thank you first of all to my supervisor, Dr. Catharine Rankin, for years of advice and opportunities and for creating a lab where students are encouraged to explore their ideas and have fun. To my lab mates, past and present, thank you for all sorts of support, I feel so fortunate to have formed lifelong friendships here. I am especially grateful to Andrew for allowing me to join and expand on this project. Tiffany, Conny, Lee, Tahereh, Andrea, and Troy, let’s keep collaborating! I have had the pleasure of working with many excellent undergraduate students as well, several have contributed directly to this project: Myron, Andy, Alex L, and Alex Y. Thank you to committee members and comprehensive examiners for guiding my research at key junctures. I have benefitted greatly from being surrounded by incredible scientists at every level of training in the UBC Neuroscience and Vancouver C. elegans communities. A special thank you to Kurt Haas and Eric Aamodt for career and science advice. This research would not have been possible without data, strains, and reagents generously shared by others, specifically the Moerman, Schafer, Lockery, Bargmann, and Xu labs.

In addition to the academic supports, many friends and family have helped me succeed (whether they knew it or not) by ensuring my life had more than the one dimension. Thank you especially to my family, Beth, Bob, Anna, and Liam, for a lifetime of support and encouragement and to the Chang’s for welcoming me into their family. Finally, thank you to my partner (best friend and psychologist), Sabrina Chang, for believing in me and inspiring me with your passionate pursuit of whatever you set your mind to.
To my Mom
1. Introduction

1.1 Habituation

Habituation is a non-associative form of learning characterized by a decremented response to repeated stimulation that cannot be explained by adaptation or fatigue. It can be observed in organisms across phylogeny and in diverse behavioral and cellular responses. It is often considered a "cognitive building-block" and the basis of selective attention. Consistent with this fundamental role, deficits in habituation are associated with a variety of neuropsychiatric disorders, including autism and schizophrenia (Kleinhans et al., 2009; Braff et al., 1992). The first scientific report of habituation (although they did not call it that) was by Peckham & Peckham in 1887. They studied an acoustic startle response of garden spiders, which dropped on a line of silk at the sound of a tuning fork, but decreased the duration of this response with repeated stimulation. Similar decreases in behaviors following repeated stimulation were reported for a wide range of organisms and assays. In a landmark paper in 1966, Spencer and Thompson outlined nine common characteristics of habituation. These were recently revised and a tenth was added (Rankin et al., 2009). The key points from each characteristic follow:

1) Repeated stimulation results in a decrease in responding to an asymptotic level (habituation) that may be preceded by facilitated responding (sensitization).

2) The response can at least partially recover (spontaneous recovery).
3) More pronounced response decrement can be induced by multiple training sessions (potentiation of habituation).
4) More rapid stimulation results in more pronounced response decrement and more rapid recovery.
5) Less intense stimulation results in more pronounced response decrement.
6) Continued stimulation at asymptotic response levels can alter subsequent behavior.
7) The response decrement shows some stimulus specificity.
8) A different stimulus can facilitate the decremented response to the original stimulus (dishabituation).
9) Repeated presentations decrease the effectiveness of the dishabituating stimulus (habituation of dishabituation).
10) Decremented responses can persist for hours, days, or weeks (long-term habituation).

Several of these characteristics are especially important for distinguishing habituation from adaptation, fatigue, or even injury. Most notably characteristic #8, that the response decrement is readily reversed by a dishabituating stimulus, highlights habituation as an attentional process. In contrast, other potential causes of decrement, such as sensory adaptation or motor fatigue, can only recover with sufficient time away from the stimulus. In addition, a more pronounced decrement to less intense stimulation and a faster recovery following more rapid stimulation (characteristics #4 and 5) is the opposite of what would be predicted for adaptation, fatigue, or injury. Finally, fatigue and injury can be ruled out if the decrement does
not generalize to another cue that elicits a similar behavior (characteristic #7). A decremented response may not display all ten features, but to qualify as habituation it is essential to rule out these other potential causes.

The aim of this dissertation is to expand our mechanistic understanding of short-term habituation. Despite its omnipresence and conservation over hundreds of millions of years of evolution, the cellular and molecular processes underlying short-term habituation remain poorly understood. Researchers have therefore turned to invertebrate model systems to help understand this fundamental phenomenon (reviewed in Kandel, 2004; Twick et al., 2014; Bozorgmehr et al., 2013). In the 1960s, Kandel and colleagues began studying learning in the marine mollusk, *Aplysia*. Touching the large shell-less sea slug’s siphon results in a protective reflex in which the siphon and gill are retracted. Repeated touching habituates this response, while a pinch to the neck or an electric shock to the tail dishabituates it (Pinsker et al., 1970). The predicted locus of plasticity is the presynaptic side of sensory neuron-to-motor neuron synapses, as habituation training does not affect the spiking of sensory cells, but decreases the number of synaptic vesicles released (Kupfermann et al., 1970; Castellucci et al., 1970; Castellucci & Kandel, 1974; Cohen et al., 1997). It is unclear what underlies the depression of the sensory neuron synapses, but several hypotheses have been put forth, including inactivation of the calcium currents required for synaptic vesicle release (Klein et al., 1980), depletion of the readily releasable pool (Gingrich & Byrne, 1985; Bailey and Chen, 1988), and silencing of individual vesicle release sites.
(Gover et al., 2002). The advantage of *Aplysia* is the ability to relate behavioral plasticity to changes at specific synapses of identified neurons.

In the 1970s, Benzer and colleagues began a genetic dissection of learning in the fruit fly, *Drosophila melanogaster*. They established an associative learning assay and conducted a forward genetic screen, identifying the first learning mutant (Quinn et al., 1974; Dudai et al. 1976). Non-associative habituation assays were subsequently developed for a variety of stimuli (i.e. visual, chemical, and mechanical) and behaviors (e.g. proboscis and leg extension). Several molecular components have been implicated in habituation using multiple assays, including (i) Dunce and Rutabaga, which degrade and synthesize cAMP (respectively; Duerr & Quinn, 1982; Engel & Wu, 1996), (ii) potassium channel subunits, including Slowpoke, Shaker, Hyperkinetic, and Ether a go go (Engel & Wu, 1998; Joiner et al., 2007), (iii) kinases, including cGMP-dependent protein kinase, Foraging (Engel et al., 2000; Scheiner et al., 2004) and calcium-calmodulin dependent kinase II, CaMKII (Jin et al., 1998; Sadanandappa et al., 2013), and (iv) a synaptic vesicle-clustering phosphoprotein, Synapsin (Godenschwege et al., 2004; Sadanandappa et al., 2013).

One of the best understood habituating circuits in any system mediates the fly's attenuated avoidance of persistent volatile repellents, wherein odorant-selective projection neurons dampen their own responsivity by potentiating inhibitory interneurons (Twick et al., 2014). Cell-specific gene rescue and knockdown has demonstrated that Rutabaga/adenylyl cyclase, CaMKII, and Synapsin induce presynaptic facilitation in these interneurons, which co-release GABA and glutamate, acting on GABA<sub>A</sub> and NMDA receptors on the projection neurons (Das et
al., 2011; Sadanandappa et al., 2013). How these components and the recurrent inhibition circuit motif apply to other habituating behaviors remains to be seen, but it is certainly distinct from the depression of excitatory synapses described for *Aplysia*. There are likely many mechanistic explanations for habituation and the best chance for a thorough characterization relies on the use of genetic model organisms with tractable nervous systems.

1.2 *C. elegans*

At about the same time that Kandel was beginning his work with *Aplysia*, Sydney Brenner chose *Caenorhabditis elegans* as an ideal organism in which to study development and the nervous system (Brenner, 1974). Today, this transparent nematode is one of the world’s best-understood metazoan thanks to the ongoing work of a very productive and collaborative research community. The worm’s small size (approx. 1 mm), short life cycle (<3 d), and ease of cultivation make it well-suited for the laboratory, and its mode of reproduction is ideal for genetic analysis, as self-fertilizing hermaphrodites can be easily inbred or crossed with males. Furthermore, the worm’s transparency grants easy access to cells for imaging, optogenetics, and ablation. Morphologically, *C. elegans* is relatively simple and its development is highly deterministic. As a result, the complete cell lineage and neural wiring diagram was elucidated; each adult hermaphrodite has only 959 cells, 302 of which are neurons forming about 5000 chemical synapses, 600 gap junctions, and 2000 neuromuscular junctions, the locations of which are largely consistent between animals (Sulston & Horvitz, 1977; Sulston et al., 1983; White et al., 1986).
*C. elegans* is currently the only organism with a completed wiring diagram of the nervous system.

*C. elegans* was the first multi-cellular organism to have its genome sequenced. Released in 1998, the 100 million base pair sequence has now been extraordinarily well annotated (Gerstein et al., 2010). After almost 50 years of random and more recently targeted genetic lesions, there is a vast library of mutant strains that can be conveniently stored as frozen stocks, with loss-of-function alleles available for an estimated 2/3 of the 20,514 protein-coding genes (Thompson et al., 2013). In addition to mutants, there are bacterial libraries available for targeting most genes through systemic RNAi by feeding (Timmons & Fire, 1998; Kamath et al., 2003). Importantly, it is estimated that 40% of these genes have human orthologs (Shaye & Greenwald, 2011). Although the specific neural circuits and behaviors differ between worms and humans, the cellular processes of plasticity are likely to be largely conserved across phylogeny.

With its invariant cell lineage and reproducible connectome, *C. elegans* was initially viewed as a genetically hardwired automaton. The deterministic development of the worm’s nervous system would seem to limit its usefulness as a model to study behavioral plasticity, but the worm has demonstrated its extreme sensitivity to experience. *C. elegans* can learn the environmental features that predict good food, bad food, no food, or aversive stimuli, allowing worms to chemotax, thermotax, or aerotax to more favorable environments. It seems that every sensory modality studied can mediate learning. Thus, its deterministic development becomes its greatest asset, as researchers can study behaviors in a
population of animals with essentially the same nervous system. The power of *C. elegans* as a genetic model has led to considerable insights into the cellular and molecular mechanisms underlying learned behaviors.

1.3 *C. elegans* habituates

1.3.1 Mechanosensory habituation

Rankin et al. (1990) were the first to characterize learning and memory in *C. elegans*. They studied plasticity of the reversal response elicited by non-localized mechanosensory input from a tap to the side of the Petri plate. The magnitude of the tap withdrawal response is around 1 mm (roughly the length of the animal), but this can change in a manner consistent with the characteristics of habituation outlined by Spencer & Thompson (1966). Repeated taps attenuates both the likelihood of reversing and the magnitude of the response (characteristic # 1; Rankin et al., 1990). This decrement spontaneously recovers over several minutes (characteristic #2; Rankin & Broster; 1992) or a brief electric shock can be used as a dishabituating stimulus to facilitate responding towards baseline levels (characteristic #8; Rankin et al., 1990). The decrement is stimulus specific, as repeated tapping does not affect the reversal response elicited by noxious heat (characteristic #7; Wicks & Rankin, 1997). The decrement is more pronounced in a second habituation session one hour after initial habituation (characteristic # 3; Lau et al., 2013; Rankin, 2000). Shorter intervals between tap (10s versus 60s) results in a faster and deeper decrement (characteristic #4; Rankin & Broster; 1992), as does decreasing the force of the tap (characteristic # 5; Timbers et al., 2013). Continued tapping at the asymptotic
response level delays the onset of spontaneous recovery, but not its rate (characteristic #6; Rankin and Broster; 1992) and with the appropriate training regime (e.g., 20 taps at a 60s interval over four sessions with 1 h rest periods between each) a decremented response can persist for up to 48h (characteristic #10; Beck & Rankin, 1995; Rose & Rankin, 2006). This thorough behavioral characterization greatly facilitated investigations into the underlying cellular processes of tap habituation.

Laser ablation was used to define the neuronal circuitry underlying the tap-withdrawal response. Chalfie et al. (1985) had previously used laser ablation to determine which neurons were mediating the worm’s reversal response to anterior touch and its forward acceleration response to posterior touch. The tap activates both the anterior and posterior mechanosensory neurons and using the circuits described by Chalfie et al. (1985) in conjunction with the neural wiring diagram (White et al. 1986), Wicks and Rankin (1995) identified the mechanosensory cells (ALM, AVM, PLM, and PVD) and interneurons (AVD, AVA, AVB, PVC, and DVA) mediating the tap withdrawal response (Fig. 1.1). Ablation of the posterior touch receptor neurons (PLML and PLMR) facilitated reversal responses, while ablation of the anterior touch receptor neurons (ALML, ALMR and AVM) promoted forward accelerations in response to tap. Thus, the tap withdrawal response arises from an integration of two competing subcircuits. By ablating the anterior or the posterior touch receptor neurons, Wicks and Rankin (1996) showed that the behavioral output of each subcircuit habituates with repeated stimulation. Importantly, the
acceleration and reversal responses habituate with distinct kinetics that integrate in a manner consistent with tap habituation in the intact animal.

In 2003, Suzuki et al. demonstrated that repeated activation could alter the response properties of the touch receptor neurons. Using a genetically encoded calcium reporter they found that repeatedly poking the anterior of the worm with a glass probe causes a cell-wide reduction in calcium response in the anterior touch receptor neuron, ALM. A similar reduction in calcium response follows repeated stimulation of the posterior touch cell, PLM (Kindt et al., 2007). Thus, attenuation of touch cell excitability with repeated activation correlates with decreased behavioral responding to tap. To test if habituation arose from a desensitization of the mechanoreceptor, O’Hagan et al. (2005) used whole-cell patch-clamp recording to measure mechanoreceptor currents in the posterior touch cell (PLM) of larval animals. They found that repeatedly poking the cell body with a glass probe had no effect on the touch-evoked mechanoreceptor current. This finding suggests that the loci of plasticity are downstream of mechanotransduction. Consistent with this hypothesis, bypassing mechanotransduction does not block habituation, as the reversal response elicited by optogenetic activation of the touch cells undergoes a robust decrement with repeated photostimulation (Nagel et al., 2005; Leifer et al., 2011; Timbers et al., 2013).

Mutant analysis of candidate genes has led to some mechanistic insights into habituation of the tap withdrawal response. Rankin and Wicks (2000) reported the first learning mutant for this assay, eat-4. EAT-4, the C. elegans ortholog of the mammalian vesicular glutamate transporter (VGLUT1), is expressed in 38 of the 118
anatomically defined neuron classes, including the touch cells underlying the touch withdrawal response (Serrano-Saiz, et al., 2013). A loss-of-function eat-4 mutant had a wild-type initial response to tap, but habituated more quickly at both a 10 and 60s interstimulus interval and failed to dishabituate following a brief electric shock. This suggests that modulation of glutamate release is an important component of mechanosensory habituation and dishabituation. Upstream of glutamate release, Cai et al. (2009) identified a K+ channel (KHT-1) and an accessory subunit (MPS-1) with a potential role in regulating attenuation of sensory cell excitability. Expressed in the anterior (ALM) and posterior (PLM) touch receptor neurons (Bianchi et al. 2003), mps-1 encodes a single-pass transmembrane protein belonging to the vertebrate KCNE family that modulates pore-forming K+ channels. Loss of mps-1 function decreases tap-withdrawal responses, a phenotype that can be rescued by expressing either wild-type MPS-1 or a variant with an inactivated kinase domain (Cai et al. 2009). Although they have a wild-type response, the worms with inactivated MPS-1 are deficient in habituation to tap at both short and long interstimulus intervals, requiring 10 times more taps to habituate and recovering almost instantaneously. Cai et al. (2009) showed that MPS-1 kinase activity inhibits KHT-1 K+ currents and that the two proteins form a complex in touch receptor neurons. They proposed that repeated activation of the touch cells results in autophosphorylation of the KHT-1–MPS-1 complex, thus diminishing K+ flux and prolonging the duration of mechanoreceptor potentials. This is predicted to slow recovery of EGL-19 (the L-type calcium channel mediating touch-evoked calcium
currents; Suzuki et al. 2003), thereby dampening cell excitability. How the kinase activity of MPS-1 is activated by repeated mechanical stimulation is unknown.

The kinetics of habituation to tap is dependent upon the context in which the stimuli are given. In the laboratory, worms are reared on agar Petri plates with a bacterial food source. When tested in the absence of the bacteria, the proportion of worms responding to tap decreases more rapidly with repeated stimulation at a 10s interstimulus interval i.e., worms habituate to tap faster when stimulated off of food (note that in this case the effect is on response frequency, there was no modulation of response magnitude; Kindt et al., 2007). Mutants in which dopaminergic signaling is disrupted habituate to tap with the rapid kinetics of wild-type worms tested off of food (Sanyal et al. 2004; Kindt et al. 2007). It was hypothesized that the texture of bacterial lawns stimulates dopamine release, which alters the functional properties of the touch cells through the D1-like dopamine receptor, DOP-1 (Sawin et al., 2000; Sanyal et al., 2004; Kindt et al., 2007). Indeed, calcium-imaging experiments in various mutant backgrounds revealed that dopamine slows the decrement of touch-evoked calcium currents in ALM via intracellular calcium release and PKC activity downstream of a Gq/PLC- β signaling cascade (Kindt et al. 2007). Thus, more rapid habituation kinetics in the absence of food correlates with more rapid attenuation of ALM excitability.

The molecules described thus far have been implicated in tap habituation as candidate genes. To identify novel pathways mediating tap habituation, Xu et al. (2002) conducted a forward genetic screen, identifying several slow habituating strains, although the causative mutations remain unknown. A genetic screen for
chemosensory adaptation (Colbert & Bargmann, 1995) identified a yet uncloned gene, *adp-1*, which has subsequently been shown to exhibit a tap habituation phenotype (Swierczek et al., 2011), suggesting alleles identified in other such screens may shed light on habituation of the tap withdrawal response. As part of his PhD thesis, Dr. Andrew Giles characterized tap habituation in 522 strains which each harbored an identified mutation in a gene predicted to function in the nervous system. By quantifying habituation rate and asymptotic level of responding for multiple behavioral metrics, including proportion of animals reversing and their response duration and speed, dozens of strains were identified with altered habituation. Notably, the habituation measures and behavioral metrics were genetically dissociable, i.e. there were strains with deficits specific to habituation rate or final asymptotic level for one or more of the behavioral metrics (reversal probability, duration, or speed). Further epistatic and cell-specific rescue experiments are needed, but a picture is beginning to emerge of the processes underlying habituation of the tap withdrawal response. Despite being touted as the “simplest” form of learning, habituation is mediated by multiple mechanisms and sensitive to training protocol and context. Some of the underlying processes are expected to be conserved across phylogeny, while others may be *C. elegans* or even circuit specific.

1.3.1 Chemosensory habituation

In addition to the tap withdrawal response, other *C. elegans* behaviors have been shown to habituate. Colbert and Bargmann (1995) observed that continuous
exposure of *C. elegans* to an attractive odorant eventually results in a loss of chemotactic response to that odorant. The molecular mechanisms underlying decremented chemosensory responding have been intensely investigated and consist of several signaling cascades dependent on the odorant, neuron, and assay (L’Etoile & Bargmann, 2000; L’Etoile et al., 2002; Kuhara et al., 2002; Matsuki et al., 2006; Palmitessa et al., 2005; Hirotsu & Iino, 2005; Miyahara et al., 2004; Kaye et al., 2009; Juang et al., 2013; O’Halloran et al., 2009; Lee et al., 2010). In most cases, chemosensory adaptation has not been distinguished from habituation. To qualify as habituation, the decremented response to the chemical cue (characteristic of habituation #1) should be readily reversed following a novel or noxious stimulus, that is, by a dishabituating stimulus (characteristic of habituation #8). Thus, a habituated animal can still sense the stimulus but is not responding, whereas an adapted animal cannot sense the stimulus until it is removed and sufficient time has passed for recovery. Bernhard and van der Kooy (2000) demonstrated that worms both habituate and adapt to odorants. First, they showed that chemotaxis to a point source of diacetyl is diminished by pre-exposure to 0.001% or 100% diacetyl, but not 0.01% or 25%. A dishabituating stimulus (centrifugation) returns to baseline the decremented response of worms pre-exposed to 0.001% diacetyl, but not those pre-exposed to 100% diacetyl, suggesting that worms habituate to 0.001% diacetyl and adapt to 100% diacetyl. Prolonged exposure to benzaldehyde also results in decreased attraction, however the decrement is inhibited in the presence of food (Nuttley et al., 2002), suggesting a learned association, making worms less likely to ignore benzaldehyde if it is predictive of an appetitive stimulus. This behavior
begins to blur the lines between associative and context dependent, non-associative learning (Pereira & van der Kooy, 2012).

It is essential to probe multiple circuits to evaluate the generalizability of mechanistic insights. The propensity to move in a chemical gradient is a higher order behavioral metric than directly quantifying the response to a discrete stimulus, like a plate tap. *C. elegans* use at least two distinct behavioral strategies for chemotaxis: (1) klinokinesis, a biased random walk with large and randomly oriented turns to promote directional change (Pierce-Shimomura et al., 1999) and (2) klinotaxis, a continuous adjustment with small and directed turns to align with the gradient (Iino & Yoshida, 2009). Studies typically just compute a chemotaxis index based on the distribution of animals in a gradient at a single time point, as opposed to deconstructing locomotion into the component sub-behaviors. However, despite the differences in modality and behavioral metric, at least one learning mutant identified with a chemotaxis assay (*adp-1*), also had a tap habituation deficit (Colbert & Bargmann, 1995; Swierczek et al., 2011). I chose to investigate habituation of the reversal response elicited by the polymodal nociceptor, ASH. ASH elicits a similar response as the touch receptor neurons that detect tap. However ASH senses diverse and even lethal stimuli and I therefore anticipated some similarities and some differences underlying the plasticity of these partially overlapping circuits.
1.4 ASH: Polymodal nociceptors

1.4.1 Molecules, circuits, behaviors

Detecting and appropriately responding to noxious stimuli is of critical importance to the survival of all organisms. Mechanisms of plasticity in these neural circuits are thus likely to have evolved early and be conserved across phyla. Cells that detect aversive or painful stimuli are called nociceptors. Many nociceptors are unusual sensory receptors in that they respond to sensory input in more than one modality, i.e. chemical, mechanical, and thermal stimuli in the noxious range (Bessou and Perl, 1969; Ochoa and Torebjork, 1989). These polymodal nociceptors may be expected to utilize unique strategies to modulate responses to diverse and salient sensory inputs. *C. elegans* has neurons that are functionally analogous to mammalian polymodal nociceptors. One class consists of the bilaterally symmetrical ASH neurons, ASHL and ASHR. Their cell bodies are in the lateral ganglia of the head and they project dendritic processes to the worm’s principle sensory organ, the amphids. Situated on either side of the mouth, each amphid is composed of two non-neuronal support cells and the ciliated endings of 12 neurons, eight of which (including ASH) make direct contact with the environment through a pore in the cuticle. Here ASH detects a variety of aversive stimuli, including several volatile and water-soluble compounds, as well as osmotic pressure and physical contact (Hilliard et al., 2005; Kaplan and Horvitz, 1993; Bargmann et al., 1990). Activation of ASH by these aversive stimuli elicits a rapid withdrawal response that often ends with a large directional change known as an omega turn. Habituation of ASH-mediated avoidance behaviors is the focus of this dissertation. I will first introduce the cellular
and circuit mechanisms underlying the response itself and follow with a discussion on its plasticity.

Several assays have been developed to study avoidance behavior mediated by ASH. For a nose touch response, an eyelash is laid in front of a crawling animal so the tip of the nose contacts the hair at a 90° angle (Kaplan and Horvitz, 1993). For volatile compounds, a hair dipped in the repellent is held at the nose (without contacting it) until the worm crawls backward (Chao et al., 2004). For chemo-avoidance of water-soluble repellents, a small (nl) drop of the compound is dripped on or in front of a crawling worm (Hilliard et al., 2002). These methods directly assay the reversal response of individual animals, but there are also indirect measures of avoidance behavior that can be applied to populations. Chemotaxis assays can be used to quantify the propensity of the population to move down a volatile or soluble repellent gradient (Troemel et al., 1997) or for soluble repellents, the propensity of the population to avoid quadrants containing the compound (Wicks et al., 2000). A barrier of soluble repellent can also be “painted” on the agar surface and the rate of crossing assayed. The barrier may separate the worms from some attractant or simply enclose a small area (Culotti & Russell, 1978; Bargmann et al., 1990). Individual and population assays have been used to identify cells and molecules required for ASH-mediated avoidance.

Laser ablation studies first implicated ASH in avoidance behavior to a variety of aversive stimuli, with secondary sensory neurons usually facilitating or sometimes antagonizing the response. For example, the residual nose touch response in animals lacking ASH is nearly completely abolished by the loss of FLP
and OLQ mechanosensory neurons (Kaplan and Horvitz, 1993), while AWB and ADL olfactory neurons contribute to the ASH-mediated escape response from volatile repellent 1-octanol (Chao et al., 2004). In contrast, reversals elicited by ASH-sensed SDS detergent are antagonized by activation of chemosensory neurons in the tail, PHA and PHB (Hilliard et al., 2002). The role of ASH as a polymodal nociceptor is highly conserved over evolutionary time, as the anatomically similar pair of neurons in other free-living and even parasitic nematodes is also required for avoidance of a variety of aversive stimuli (Srinivasan et al., 2008; Ketschek et al., 2004; Forbes et al., 2004). While laser ablation and cell-specific mutant rescue studies demonstrated the necessity of ASH in many avoidance responses, direct depolarization demonstrated its sufficiency, inducing reversals with all the hallmarks of the natural response. This was first shown by expression of a leaky glutamate receptor in ASH, which approximately doubled the rate of reversals (Zheng et al., 1999). Ectopic expression of the mammalian cation channel, TRPV1, allowed for drug-inducible activation of ASH with capsaicin, which elicits a rapid reversal response (Tobin et al., 2002). More recently, optogenetic activation of ASH has been used to simulate aversive stimuli with light (Guo et al., 2009; Ezcurra et al., 2011; Husson et al., 2012).

The final step in defining ASH as a polymodal nociceptor was to monitor cellular activity with exposure to naturally occurring cues. Expressing a genetically encoded calcium indicator in ASH, Hilliard et al. (2005) observed rapid increases in intracellular calcium at the soma in response to a variety of noxious stimuli, including quinine, denatonium, SDS, copper, glycerol, distilled water, and nose
touch. These responses were unaffected by the loss of a gene required for synaptic release \((\text{unc-13})\), implicating ASH as the primary sensor. The length of the cellular response increased with exposure duration to repellents, with calcium levels peaking after a few seconds and slowly declining towards baseline over 30s. This suggests that ASH responds to the presence of cues, as opposed to changes in concentration, but adapts during prolonged exposure. Chronis et al. (2007) observed the sustained calcium response during copper or glycerol exposure, but also noted a transient increase in calcium levels upon stimulus removal. Using GCaMP3, Kato et al. (2014) demonstrated that ASH neurons can respond to subsecond stimulus fluctuations, but the ability to sustain these responses varied across stimuli, with responses to high osmolarity \((\text{glycerol and NaCl})\) persisting longer than responses to copper, quinine, or dihydrocaffeic acid.

ASH expresses genes specific to the different modalities it detects. In some cases the sensory transduction machinery has been identified. For example, the DEG/ENaC protein DEG-1 is the major mechanotransduction channel (Geffeney et al., 2011), TMC-1 is the sodium-sensitive ion channel for salt sensation (Chatzigeorgiou et al., 2013), the G protein–coupled receptor SRI-14 is an olfactory receptor for diacetyl (Taniguchi et al., 2014), and the G protein–coupled receptor, DCAR-1, is the candidate receptor for water-soluble repellent dihydrocaffeic acid (Aoki et al., 2011). Modality-specific pathways also mediate downstream signal transduction, for example the novel cytosolic protein OSM-10 is essential for the behavioral and cellular response to osmotic shock, but not nose touch or volatile or water-soluble repellents (Hart et al., 1999; Hilliard et al., 2005), while the inositol
1,4,5-trisphosphate receptor, encoded by *itr-1*, is essential for avoidance from nose touch and benzaldehyde, but not osmotic shock (glycerol or fructose) or volatile or water-soluble repellents (Walker et al., 2009), and finally, the novel WD-40 repeat protein QUI-1 is required for avoidance of volatile and water-soluble repellents, but not nose touch or osmotic shock (Hilliard et al. 2004; Fukuto 2004). Modality-specific signaling downstream of sensory transduction remains poorly understood.

Signal transduction for ASH-sensed stimuli converges on the TRPV-related cation-selective channel partners, OSM-9 and OCR-2, which co-localize to the apical cilia of ASH. Originally identified in a forward genetic screen for osmotic shock insensitivity, OSM-9 has since been shown to be essential for the behavioral and cellular (calcium transients at soma) responses to most, if not all, ASH-sensed stimuli (Colbert et al, 1997; Tobin et al, 2002; Hilliard et al., 2005). TRP channels have also been linked to pain sensation in mammals (Levine & Alessandri-Haber, 2007) and expression of mammalian TRPV4 in ASH was able to rescue some, but not all of the deficits associated with loss of OSM-9 – the TRPV4 transgene restored sensitivity to osmotic and mechanical inputs, but not to a volatile repellent (Liedkte et al., 2003). Polyunsaturated fatty acids are thought to comprise the upstream signaling cascade activating OSM-9/OCR-2 channels (Kahn-Kirby et al., 2004). Consistent with a role in initiating cell excitation, OSM-9 is dispensable for reversal responses elicited by direct depolarization of ASH in transgenic animals using capsaicin to activate mammalian TRPV1 or blue light to activate the light-gated cation channel, Channelrhodopsin-2 (ChR2; Tobin et al., 2002; Guo et al., 2009). In contrast, responses elicited by these direct depolarization approaches are
dependent on more downstream processes required for signal transmission, like the vesicular glutamate transporter encoded by eat-4 (Tobin et al., 2002; Guo et al., 2009). I will now describe the cells and circuits to which ASH signals.

Based on the wiring diagram and laser ablation of candidate neurons, the bilaterally symmetrical AVA, AVD, and AVE neurons were hypothesized to be the command interneurons that drive backward locomotion via electrical and chemical output onto the excitatory cholinergic A and B motor neurons that control body wall muscles (White et al., 1976; Chalfie et al., 1985; Wicks et al., 1996). Consistent with this model, calcium increases in AVA and AVE temporally correlate with backward locomotion (Ben Arous et al., 2010; Chronis et al., 2007; Kawano et al., 2011) and their optogenetic activation elicits backwards locomotion (Schmitt et al., 2012; Stirman et al., 2012). ASH has direct chemical synapses onto the reverse command interneurons, with a total of 6 onto AVA, 9 onto AVD, and 3 onto AVE (Fig. 1.1; White et al., 1986). Laser ablation of reverse command interneurons decreases the likelihood of responding to ASH-sensed stimuli (Piggott et al., 2011). Functional ablation in adults has ruled out developmental deficits associated with larval cell ablation. Responses to osmotic shock were impaired by hyperpolarization of the reversal command interneurons using histamine treatment on transgenic worms with cell-specific expression of a Drosophila histamine-gated chloride channel (Pokala et al., 2014), while optogenetic hyperpolarization of command interneurons impaired responding to optogenetic activation of ASH (Husson et al., 2012). The functional connection between ASH and AVA has been quite well studied. Voltage-clamping AVA, Mellem et al. (2002) were able to record inward synaptic currents
following a nose touch, which activates ASH in addition to mechanosensitive FLP and OLQ neurons. In an all-optical approach, Guo et al. (2009) used a high-power epifluorescence light source and a digital-light-processing mirror array to specifically excite ChR2 in ASH while simultaneously monitoring calcium levels via G-CaMP activity with low intensity and intermittent 488 nm laser light, thus preventing crosstalk with ChR2 (which has an excitation spectrum that overlaps with G-CaMP). Using this setup, they observed evoked calcium transients in AVA and AVD that were dependent on the vesicular glutamate transporter expressed in ASH, EAT-4 (Lee et al., 1999). Combining the approaches, Lindsay & Lockery (2011) used photo-electrophysiology to record synaptic currents in AVA evoked by optogenetic activation of ASH. By adjusting irradiance intensity, they were able to demonstrate graded synaptic connectivity between ASH and AVA. The currents were partially blocked by simultaneous application of glutamate antagonists CNQX and MK-801. While most other sensory neurons of the amphid signal through one or two layers of interneurons, ASH is directly connected to the reversal command interneurons, allowing for rapid escape responses.

In addition to the stimulatory circuit mediated by the reversal command interneurons AVA, AVD, and AVE, Piggott et al. (2011) propose a parallel disinhibitory circuit in which ASH activation of AIB inhibits the second-layer interneuron pair, RIM, relieving the tonic suppression of reversals that occurs during locomotion (Fig. 1.1). Consistent with this model, laser ablation of both the stimulatory and disinhibitory pathways decreased the probability of nose touch and osmotic avoidance reversal responses even more than loss of either pathway alone.
Monitoring calcium concentration in freely moving worms, Piggott et al. (2011) observed a calcium efflux in RIM in response to nose touch, but an influx in response to osmotic shock. The difference between the stimuli may be mediated by modality-specific output of ASH or the secondary sensory neurons recruited for nose touch versus osmotic shock. Interestingly, reversal responses could be elicited by inhibition of RIM with halorhodopsin or by activation of RIM with ChR2 (Guo et al., 2009; Piggott et al., 2011). Animals lacking AVA, AVD, and AVE still reversed to RIM inhibition, but did not reverse to RIM activation, suggesting that the stimulatory and disinhibitory pathways are parallel circuits with cross-talk. Recording voltage signals through current clamp, a nose touch was seen to elicit a depolarizing signal in ASH, AVA, and AIB, but a hyperpolarizing signal in RIM that was dependent on AIB. Consistent with hypotheses from earlier behavioral analysis (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002), the EPSPs in AVA and AIB required GluR1 homolog, GLR-1, while the IPSP in RIM was mediated by the glutamate-gated Cl⁻ channel subunit encoded by avr-14. While RIM activation likely elicits reversals via AVA, it is unclear how the disinhibitory circuit promotes backward crawling. It is tempting to speculate though that it occurs as a result of RIM’s connection with the forward command interneuron AVB. It is important to note that several other interneurons are well positioned to modulate both the stimulatory and disinhibitory circuits.
1.4.2 Modulation

The focus of this dissertation is the plasticity of the ASH-mediated avoidance response. There are currently three factors known to modulate this response, quiescence, feeding state, and prior exposure.

Quiescence

Behavioral quiescence is a sleep-like state characterized by decreased locomotion and feeding. It occurs during the lethargus period that precedes larval molting and with satiety in adults (Van Buskirk & Sternberg, 2007; You et al., 2008). It can also be induced by overexpression of EGF-like ligand LIN-3 (Van Buskirk & Sternberg, 2007). Quiescent animals are slow to respond to ASH activation, but this can be reversed if the ASH-sensed stimulus is preceded by mechanical stimulation to “wake” worms up (Raizen et al., 2008; Cho & Sternberg, 2014). Monitoring calcium currents in the ASH avoidance circuit, Cho & Sternberg (2014) demonstrated that ASH responded less to both copper and glycerol during lethargus, as did the downstream reversal command interneurons AVA and AVD. In addition, responding in AVA and AVD lose synchrony during lethargus. Importantly, prior mechanical stimulation restored behavioral responding, as well as coordination of AVA and AVD, without affecting responsivity of ASH. This demonstrates that the ASH avoidance circuit is modulated at the sensory, as well as the interneuron level to delay responses during lethargus.
Feeding state

Feeding state affects a number of *C. elegans* behaviors and the presence of food has also been shown to alter the worm's reversal response to stimuli sensed by ASH. Dopamine and serotonin are neuromodulators regulating many *C. elegans* food-related behaviors. Ezcurra et al. (2011) showed that reversal responses to three different water-soluble repellents sensed by ASH (CuCl₂, glycerol, and primaquine) are more likely if animals are tested in the presence of food or just exogenous dopamine, but not exogenous serotonin. As predicted, a dopamine deficient cat-2 mutant behaves on food as though tested in its absence, a phenotype that can be rescued by the introduction of exogenous dopamine. Reversal responses can also be promoted off of food by optogenetic activation of the dopaminergic, but not the serotonergic neurons. Imaging calcium transients in ASH, Ezcurra et al. (2011) showed that food and dopamine potentiate responses to water-soluble repellents. An invertebrate-specific dopamine GPCR, DOP-4, is required in ASH for dopamine's influence on both the cellular and behavioral response.

Modulation of ASH avoidance behavior is somewhat modality-specific. In stark contrast to water-soluble repellents, disrupted dopamine signaling actually facilitates responses to a volatile repellent detected by ASH, i.e. reaction time to octanol is accelerated in a dopamine deficient cat-2 mutant (Ferkey et al., 2007; Wragg et al., 2007). This effect is mediated by DOP-3 (a D2-like dopamine GPCR) signaling in the sensory neuron (Ezak & Ferkey, 2010). The time to initiate a reversal to dilute octanol is however delayed in the absence of food (Chao et al., 2004), approximately doubling from 5 to 10s. In addition to the slowed reaction
time, Harris et al. (2011) observed that the responses are larger and more likely to result in a trajectory change. Like dopamine, serotonin can also signal the presence of food. Animals unable to synthesize serotonin respond to octanol on food as though tested in its absence, a deficit that can be ameliorated by supplementing serotonin (Chao et al., 2004; Harris et al., 2011). C. elegans has at least nine serotonergic neurons and Harris et al. (2011) identified the NSM pair as a key source of serotonin for food modulation of octanol avoidance. Three different serotonin GPCRs have been implicated, SER-5 functions in ASH, while MOD-1 and SER-1 function in downstream interneurons (Harris et al., 2009; Zahratka et al., 2014). In ASH, serotonin is hypothesized to facilitate avoidance behavior by promoting the release of neuropeptides encoded by nlp-3, which genetic, but not biochemical, evidence suggests signal through NPR-17 (Harris et al., 2010). Zahratka et al. (2014) recently demonstrated that serotonin potentiates octanol-evoked depolarization of ASH, but reduces somal calcium transients. Food and serotonin also promote nose touch responding (Chao et al., 2004), but serotonin does not alter touch evoked calcium transients at the ASH soma (Ezcurra et al., 2011; an earlier study by the same group had reported that the cellular response was dependent on serotonin; Hilliard et al., 2005). Food modulation of the avoidance response is therefore surprisingly complex, dependent on both the modality and intensity of the stimulus.

The stimulatory effects of food and serotonin on octanol avoidance are counteracted by both octopamine and its biosynthetic precursor tyramine, the monoamines thought to transmit the starvation signal. Octopamine is often
considered the invertebrate counterpart to norepinephrine based on its structural similarity and homology of its receptors. The biosynthetic enzyme required for octopamine synthesis, tyramine β-hydroxylase (TBH-1) is expressed in a single pair of interneurons, RIC (Alkema et al., 2005). The biosynthetic enzyme required for tyramine synthesis, tyrosine decarboxylase (TDC-1), is additionally expressed in the RIM interneuron pair (Alkema et al., 2005). Despite their limited release sites, octopamine and tyramine activate global peptidergic signaling cascades to inhibit octanol avoidance (for review see Mills et al., 2012; Komuniecki et al., 2012). In the model, tyramine’s effect depends on GPCR TYRA-3 in ASI (Hapiak et al., 2013), while octopamine acts on two GPCRs expressed in ASH, OCTR-1 and SER-3, and also on the SER-6 GPCR expressed in at least three other sensory neurons, AWB, ADL, and ASI (Wragg et al., 2007; Mills et al., 2012). Stimulation by octopamine and tyramine is thought to promote the release of neuropeptides from AWB (NLP-9), ADL (NLP-7, NLP-8), and ASI (NLP-1, NLP-6, NLP-7, NLP-9) that ultimately signal to several neurons to inhibit the initiation of reversals.

Prior exposure

While there is a growing literature on its modulation by food, considerably less is known of the mechanisms by which experience alters the ASH avoidance circuit. This is the primary focus of this dissertation. In 1999, Hart et al. reported that administering 40 nose touches in rapid succession decreased the likelihood of responding. This prior mechanosensory stimulation to the nose did not affect the time to reverse to octanol or the degree to which worms were retained by an
osmotic ring. Similarly, the time to respond to octanol more than tripled after two 15s exposures to it, but pre-exposure to octanol had no observable effect on the likelihood of a nose touch response. Thus, the decrement to persistent aversive cues detected by ASH was at least partially stimulus-specific. For water-soluble repellents, Hilliard et al. (2005) demonstrated a rapid decrease in the probability of responding to CuCl₂ over 10 stimuli administered at a 10s interval. Responses mostly recovered over 5min. A similar decrement was induced by a 1 min prolonged exposure, which had minimal, if any effect on the probability of responding to glycerol, again suggesting some degree of stimulus-specificity. Monitoring calcium currents in ASH, Hilliard et al. (2005) showed that the behavioral decrement correlated with a decrease in cellular responsiveness and that this was also true in a mutant lacking synaptic release (unc-13). Therefore, ASH displays a cell-intrinsic and reversible decrement in responding with repeated or prolonged exposure to a water-soluble repellent. GPC-1, one of two C. elegans G-protein γ - subunits, had previously been implicated in behavioral adaptation to soluble attractants (Jansen et al., 2002), prompting Hilliard et al. (2005) to test its role in ASH-mediated responding. For soluble repellents, the degree of decrement was also reduced by its loss, both in terms of the ASH cellular response and behavioral avoidance. Another mechanistic insight comes from a recent study by Lindy et al. (2014) showing that the behavioral decrement associated with prolonged exposure to hypertonicity depends on the ability of TRPV-like channel OSM-9 to flux calcium. To examine the structure-function relationship of the OSM-9 selectivity filter, Lindy et al. (2014) rescued OSM-9 expression in ASH using several mutagenized variants. OSM-9 Y604F
was especially interesting because it did not flux calcium, but still supported 
behavioral responding, indicating another cation (presumably Na+) could drive 
avoidance behavior. Although their naïve response was intact, worms expressing 
Y604F differed from wild-type in that their responses remained robust after 
prolonged exposure to hypertonicity. Therefore the OSM-9 calcium flux is 
dispensable for ASH activation, but essential for plasticity of the response. How 
calcium and GPC-1 support decremented responding remains to be determined. 
Decreased cellular responsivity to specific stimuli suggests that ASH can adapt, 
however it is unclear if the avoidance circuit can also habituate to repeated stimuli. 
Furthermore, the behavioral metric typically scored in these assays is response 
probability and it is unknown what other behavioral plasticity is associated with 
persistent exposure to aversive stimuli.

As with tap habituation, food promotes responding to repeated ASH 
activation. As described above, food is known to affect naïve responding, but with a 
sufficiently intense stimulus, modulation by food is only apparent with persistent 
exposure. Specifically, Ezcurra et al. (2011) showed that the probability of 
responding to 10 mM CuCl2 was initially unaffected by feeding state, but with 
repeated or prolonged exposure responses persisted longer in the presence of food. 
Calcium imaging in ASH correlated with the behavioral data, suggesting food is 
acting upstream of cell excitability to promote responding following persistent ASH 
activation. Exogenous dopamine mimicked the presence of food for behavioral and 
cellular responses and a dopamine-deficient cat-2 mutant behaved on food as if 
tested in its absence.
1.5 Conclusion

Habituation is a fundamental form of learning that remains poorly understood at the cellular and molecular level. *C. elegans* is an excellent model system in which to investigate this highly conserved phenomenon. The escape response elicited by polymodal nociceptors ASHL and ASHR is well studied at the molecular, cellular and circuit levels. ASH is interesting in the context of habituation because of the diversity and salience of the cues it detects, including some that are potentially lethal for *C. elegans*. Parallels can be drawn between this circuitry and pain sensation in mammals, most notably the importance of OSM-9, the functional homologue of mammalian TRPV4 (Liedtke et al., 2003) and the modulation by monoamines and neuropeptides (Mills et al., 2012). Previous research reported a decrement in responding to persistent ASH-sensed stimuli (Hart et al., 1999; Hilliard et al., 2005; Ezcurra et al., 2011). This is striking, as noxious sensory input should always be important to attend to. To better understand the response decrement associated with repeated ASH activation, I set out to establish a high-throughput habituation assay using real-time computer vision software for detailed behavioral tracking during learning. Once wild-type behavior is fully characterized, a high-throughput assay facilitates the discovery of key molecules using forward or reverse genetic screens. With molecular components implicated in a particular process, the tractable nervous system of *C. elegans* allows them to be placed in the underlying circuitry to reveal how sensory information is processed to affect behavior.

The objectives of my research are therefore:
1) To establish a high-throughput habituation assay for ASH-mediated responses.

2) To identify molecular components mediating behavioral plasticity.

1.6 General methods

1.6.1 Strains

Animals were maintained on nematode growth medium (NGM) seeded with *Escherichia coli* (OP50) as described in Brenner (1974). Two integrated Channelrhodopsin-2 (ChR2) transgenes (gifts from William Schafer, MRC Laboratory of Molecular Biology) were used in these studies. One transgene drove ChR2 with the *sra-6* promoter, which expresses strongly in ASH and more weakly in a pair of sensory neurons (ASI) and interneurons (PVQ; Troemel et al., 1995). The other transgene employed intersecting promoters and FLP Recombinase to specifically target ChR2 to ASH (ASHp::ChR2), as reported by Ezcurra et al. (2011) and independently confirmed by another research group (Schmitt et al., 2012). The strain with the *sra-6p::ChR2* transgene showed robust responding at irradiances well below the threshold for the *C. elegans* innate response to blue light (for behavioral experiments I used 70 μW/mm²). The ASHp::ChR2 strain required increased irradiance (I used 250 μW/mm²) and therefore experiments with this strain were performed in a background lacking LITE-1, a native *C. elegans* short-wavelength light receptor (Ward et al., 2008; Edwards et al., 2008). The increased sensitivity of the *sra-6p::ChR2* strain was most likely caused by elevated ChR2 levels in ASH, as the off-target neuron classes are not predicted to elicit rapid escape.
responses. In most cases both strains were tested and complementary results were taken as evidence for a behavioral consequence of ASH activation. The ASHp::ChR2 transgene was used for mutant analysis, except when the gene of interest was located on chromosome X, where the ASHp::ChR2 transgene was integrated (sra-6p::ChR2 was integrated on chromosome V). Throughout, “control” refers to one of two strains: AQ2235 lite-1(ce314) ljls114[gpa-13p::FLPase + sra-6p::FTF::ChR2::YFP] for experiments with the ASHp::ChR2 transgene or VG61 yvls1[sra-6p::ChR2::YFP + unc-122p::GFP] for experiments with the sra-6p::ChR2 transgene. Mutants were obtained from the Caenorhabditis Genetics Center and Japan’s National BioResource Project for the nematode.

1.6.2 Behavioral tracking

NGM plates were spread with 50-100µL E. coli OP50 liquid culture mixed with all-trans retinal (ATR; or equal volume of ethanol vehicle) for a final plate concentration of 5µM ATR. Plates were stored at room temperature in the dark for 24-48 hours before use. For age-synchronized colonies, gravid adults were left 3-6 h to lay 20-80 eggs before being removed from the plate. Animals were reared at 20°C and tested 3 or 4 days after hatching. Behavioral tracking typically occurred directly on the rearing plates, in which case the mean number of animals tracked per plate ± SEM is reported in the figure legend. Transgenic strains with extra-chromosomal arrays were reared similarly, but 30-40 animals were picked (based on expression of a fluorescent co-injection marker) to ATR-containing food plates 24h before testing, with control strains similarly handled.
Multi-Worm Tracker software (version 1.2.0.2) was used for stimulus delivery and image acquisition (Swierczek et al., 2011). Following a 3 to 5 min acclimatization phase, stimuli were presented using custom built LED rings capable of illuminating 6 mm or 3.5 mm (diameter) Petri plates from above with uniform blue light (max = 70μW/mm² or 250μW/mm², respectively). An orange filter prevented the blue light from entering the camera. Taps were delivered by an electromagnetic tubular push solenoid. Offline analysis with Choreography software (version 1.3.0_r1035; Swierczek et al., 2011) used “--shadowless”, “--minimum-time 20”, and “--minimum-move-body 2” filters with “--each-id all” and “--speed-window 0.1” output options to obtain “--bias,speed,x,y” data for all valid objects. The MeasureReversal plugin was used to identify reversals occurring within 3s (dt=3) of the light pulse onset.

1.6.3 Statistics

Custom MatLab scripts organized and analyzed the Choreography output files. Student's t-tests and one-way ANOVAs with Tukey's honestly significant difference (HSD) criterion were used to compare the responses between strains and treatments. For response probability, tests compared the mean from the proportion of worms responding on each plate (n = number of plates tested). For magnitude metrics (i.e. duration, latency, and displacement) data were combined across plates and collective means were compared (n = number of animals tested). For all statistical tests, α was 0.05.
Figure 1.1. Wiring diagram depicting the interconnectivity of the primary neuron classes underlying tap and ASH-mediated reversals. With the exception of unpaired AVM, all sensory and inter-neuron classes in the circuit comprise two cells, while there are 9 DA, 7 DB, 12 VA, and 11 VB motor neurons.
2. Role of food and dopamine in habituation

2.1 Introduction

ASH neurons are functionally analogous to mammalian polymodal nociceptors, as the pair detects a variety of aversive stimuli, including volatile and non-volatile repellents, as well as osmotic pressure and physical contact (Hilliard et al., 2002; Kaplan and Horvitz, 1993; Bargmann et al., 1990). These cells are especially interesting in the context of habituation because of the diversity and salience of the stimuli they detect. Repeated or prolonged exposure to ASH-sensed stimuli result in a decreased likelihood of responding that is mostly stimulus specific (Hart et al., 1999; Hilliard et al., 2005; Ezcurra et al., 2011). There are several limitations associated with studying habituation of ASH-mediated reversal responses using the classical aversion assays. Although ASH detects a variety of stimuli, none of them are easily administered discretely to an entire population of worms simultaneously. To investigate the reversal response directly, experiments using naturally occurring cues must be conducted one worm at a time, producing a bottleneck for large-scale studies. Furthermore, the precise timing and intensity of these stimuli are difficult to control. This is significant for learning assays, as both the interstimulus interval and the strength of the stimulus are known to affect habituation. In addition, the naturally-sensed stimuli activate secondary sensory neurons known to contribute to or antagonize the response.

In an attempt to circumvent the issues described above, I adapted a recently developed automated behavioral analysis setup, The Multi-Worm Tracker (Swierczek et al., 2011), for use in optogenetic experiments. In optogenetics, light is
used to manipulate or monitor the activity of cells expressing a genetically encoded light-sensitive protein. In nervous systems, a light-gated cation channel can be used to depolarize neurons, thus allowing precise temporal control of a genetically defined subset of cells. The nervous system of *C. elegans* is optically and genetically accessible and was one of the first to be targeted for optogenetic manipulation in awake and behaving animals (Nagel et al., 2005). In the high-throughput learning assay developed in this chapter, ASH is repeatedly photoactivated with Channelrhodopsin-2 (ChR2), a blue light-gated cation channel (Nagel et al., 2003). This approach negates the problems of previously described single-worm assays, as ASH can be depolarized in an entire population of worms simultaneously with whole-plate illumination at defined time-points and intensities. The response of dozens of worms can then be quantified automatically using the Multi-Worm Tracker, which offers an unprecedented ability to generate highly detailed behavioral data for large numbers of animals. Furthermore, using ChR2 to bypass normal sensory transduction prevents adaptation at this level and therefore behavioral plasticity can be attributed to more downstream mechanisms.

By dissecting behavior into multiple components I uncovered a suite of changes characteristic of habituation and sensitization that together acted to promote dispersal. I found that the D1-like dopamine receptor, DOP-4, modulated this dispersal as a function of feeding state.
2.2 Methods

2.2.1 Strains

The ChR2 transgenes were crossed into BZ873, CB1112, CX10, GR1321, LX636, LX702, MT8943, MT13113, RB665, RB785, RB1254, RB1680, TQ1716, TU253, and VM3109 to generate the following strains:

VG53 glr-1(ky176); yvls1
VG54 mec-4(u253); yvls1
VG224 cat-2(e1112); lite-1(ce314) ljls114
VG231 tdc-1(n3419); lite-1(ce314) ljls114
VG233 tph-1(mg280); lite-1(ce314) ljls114
VG236 dop-3(ok295); yvls1
VG237 dop-1(vs101); yvls1
VG238 dop-1(ok398); yvls1
VG240 dop-2(vs105); lite-1(ce314) ljls114
VG241 dop-5(ok568); lite-1(ce314) ljls114
VG248 dop-4(ok1321); yvls1
VG249 dop-6(ok2090); yvls1
VG284 bas-1(ad446); cat-4(e1141); lite-1(ce314) ljls114
VG388 trp-4(sy695); lite-1(ce314) ljls114
VG389 trp-4(sy695); lite-1(ce314) ljls114; xuEx584[dat-1p::trp-4-1-2-YFP + unc-122p::GFP]
VG515 osm-9(ky10); yvls1
The *dop-4* rescue plasmids (a gift from William Schafer, MRC Laboratory of Molecular Biology, and described in Ezcurra et al., 2011) were co-injected into the gonad of VG248 adult hermaphrodites with pCFJ90 (*myo-2p::mCherry; Frøkjær-Jensen et al., 2008*) for use as a visible marker and pBluescript to make the total DNA concentration 100ng/ul (Mello et al., 1991). The following strains were generated by microinjection:

VG384 *dop-4(ok1321); yvls1; yvEx96[gpa-13p::dop-4(35ng/ul) + myo-2p::mCherry(5ng/ul)]

VG394 *dop-4(ok1321); yvls1; yvEx98[dop-4p::dop-4(35ng/ul) + myo-2p::mCherry(3ng/ul)].

2.2.2 Behavioral tracking

See general methods section of Chapter 1 for details. To evaluate the effect of food, 20min prior to testing animals were picked from their rearing plates to an unseeded NGM transfer plate where they remained for several minutes to crawl away from residual *E. coli* before being transferred to a plate spread 0.5 to 2 hours earlier with either 50ul OP50 liquid culture or just the LB.

2.2.3 Nose touch

The nose touch assay was conducted on rearing plates with the experimenter blind to strain (*glt-1 + or -*) or treatment (*ATR + or -*). Nose touch sensitivity was assayed within 30s of the last light pulse. Positive responses were defined as
reversals elicited by contact with an eyebrow hair placed perpendicular to the direction of forward motion (Kaplan and Horvitz, 1993).

2.2.4 Octanol exposure

Four 3ul drops of 30% 1-octanol or the ethanol vehicle were placed on the Petri plate lid of rearing plates. The plates were sealed with parafilm and the Multi-Worm Tracker was used to monitor responses elicited by ChR2 activation.

2.3 Results

2.3.1 Population assay for repeated ASH activation

To stimulate an entire population simultaneously with precise temporal and intensity regulation I used optogenetics in combination with a real-time computer vision system, the Multi-Worm Tracker (Swierczek, et al., 2011). Using a custom built LED light ring, reversal responses to 2s blue light pulses were detected in the majority of animals with ChR2 expressed in ASH and fed the essential opsin co-factor, ATR. To examine the plasticity of this response, light pulses were administered every 10s for 5min. Figure 2.1A depicts speed profiles for 50 representative animals over the first and final 20s of the assay. Blue light illumination promoted reversal behavior, but only in the population fed ATR. Training had a variety of behavioral consequences that were quantified with several metrics. In terms of the probability of responding, the majority of animals typically reversed to each stimulus of the assay (Fig. 2.1B), however with repeated stimulation, responses were of decreased duration (i.e. time spent moving
backwards; Fig. 2.1C) and slowed reaction time (Fig. 2.1D). The ~0.5s increase in response latency could not fully account for the ~1.5s decrease in response duration, suggesting they are independent metrics.

There were also changes to locomotory behaviors during the periods between stimuli. Examining the 3s window immediately preceding a stimulus, I quantified the proportion of animals moving forward, backward, or paused. With repeated stimulation, the behavioral state of the population shifted, such that more time was spent moving forward with a decreased likelihood of either pausing or reversing (Fig. 2.2A). In addition to increased prevalence, the speed of forward movement was also increased (Fig. 2.2B). This shift in locomotory behavior was dependent on photocurrents through ChR2, as it did not occur on plates without ATR (Fig. 2.2). The overall pattern of behavioral change was the same for the strain expressing ChR2 by the sra-6 promoter.

To evaluate the spontaneous recovery from the 30 light pulses I waited intervals of varying duration (20s, 40s, 70s, or 130s) before administering a 31st stimulus. Responses had not recovered if a single stimulus was omitted (i.e. a 20s recovery), but a one-way ANOVA showed a significant difference between recovery intervals for both reversal duration ($F(3,542) = 18.5, p < 0.0001$) and latency ($F(3,542) = 4.0, p = 0.008$) metrics. Tukey’s HSD comparisons indicated that reversals had returned towards baseline over 130s, as responses after this interval were of increased duration and decreased latency as compared to responses after only a 20s recovery period ($p < 0.05$; Fig. 2.3A,B). The decrement was therefore reversible with a time constant similar to previous reports using naturally-sensed
stimuli (Hilliard et al., 2005). Monitoring locomotion over the 130s recovery period, there was also a shift toward the pre-assay distribution of forward, backward, and paused movement (Fig. 2.3C), as well as a deceleration of forward speed (Fig. 2.3D).

2.3.2 Generalization of photoactivation and naturally sensed stimuli

ChR2 photocurrents peak in milliseconds and then decay to a steady state in a phenomenon known as desensitization or inactivation (Nagel et al., 2003). Light adaptation refers to the reduction in peak current (but not steady state current) that occurs with repetitive light pulses (Lórenz-Fonfría & Heberle, 2014). If decremented behavioral responding arose from light adaptation of ChR2, then the consequence of repeated ASH photoactivation should not generalize to naturally occurring cues. I tested this hypothesis in a nose touch assay preceded by twenty 2s light pulses presented every 10s. ATR-fed ChR2 transgenic animals were less likely to reverse following a head-on collision with an eyelash (25%) than unstimulated controls (78%) or stimulated animals reared in the absence of ATR (91%; Fig. 2.4A), indicating that the change was occurring downstream of ChR2 photocurrents. The experimental group’s level of responding to nose touch was comparable to a nose touch-defective glr-1 mutant (19%; Fig 2.4A; Maricq et al., 1995; Hart et al., 1995). In a complementary experiment, a 3min exposure to a volatile repellent detected by ASH, octanol, had the same behavioral consequence as repeated ASH photoactivation, i.e. compared to vehicle, octanol pre-exposure decreased the duration ($t(198) = 6.9, p = 0.00001$) and increased the latency ($t(198) = 2.6, p = 0.01$) of reversal responses elicited by ASH photoactivation (Fig. 2.4B). As a negative
control, I tested a mutant lacking OSM-9, the TRPV-like channel required for initiating cell excitation to most (if not all) ASH-sensed stimuli. For the osm-9 mutant, responses for the octanol and vehicle pre-exposed groups were indistinguishable both in terms of duration ($t(139) = 1.3, p = 0.20$) and latency ($t(139) = 0.29, p = 0.77$). These experiments demonstrate bi-directional generalization of photoactivation and naturally sensed cues, confirming that the behavioral decrement is not simply light adaptation of ChR2 photocurrents and validating the use of optogenetics.

### 2.3.3 Dishabituation

A habituated animal can sense the persistent stimulus but is suppressing naïve responding, whereas an adapted animal cannot sense the stimulus until sufficient time has passed for recovery. Habituated responses are readily reversed in a phenomenon known as dishabituation, which distinguishes habituation from sensory adaptation or fatigue. I tested whether a tap to the side of the Petri plate could dishabituate responses following repeated photoactivation of ASH. Tap was chosen because it could be discretely applied to the entire population simultaneously. Furthermore, the interneurons and motor neurons mediating the tap-withdrawal response are mostly overlapping with those required for ASH-mediated reversals (Wicks & Rankin, 1995; Guo et al., 2009; Guo et al., 2011; Piggott et al., 2012). To test for dishabituation I repeatedly illuminated two groups at a 10s interstimulus interval for 5min and then waited 20s before administering a final 2s light pulse. One group was given a tap 10s into the 20s recovery period. This non-
localized mechanical stimulus acted as a dishabituating cue since tapped animals responded with longer reversals to the subsequent light pulse than untapped controls, $t(257) = 4.4, p < 0.00001$, which showed no appreciable spontaneous recovery over 20s (Fig. 2.5A). Although reversal duration was facilitated by tap, reaction time was not, $t(257) = 0.7, p = 0.46$ (Fig. 2.5B). This suggests that the plasticity underlying these two metrics arises from distinct processes. The effect of repeated ASH photoactivation on response latency may either represent sensory adaptation or fatigue or tap may not be an appropriate dishabituating stimulus for this metric.

The tap withdrawal response is mediated by five body touch receptor neurons located along the length of the animal (Wicks and Rankin 1995). Although ablation of ASH neurons does not impact the tap withdrawal response (Wicks and Rankin 1995), it is a mechanosensor and could potentially detect the agar vibrations. To confirm that the body touch receptor neurons are the source of the dishabituating signal, a mec-4 loss-of-function mutation was used to specifically render them insensitive to mechanical stimuli (O’Hagan et al., 2005). Comparing reversal duration for the first, final, and dishabituated light pulse, a one-way ANOVA showed a significant difference for both control ($F(2,694) = 205.9, p < 0.00001$) and the mec-4 mutant ($F(2,592) = 89.5, p < 0.00001$; Fig. 2.5C). However, Tukey’s HSD comparisons indicated that the final and dishabituated responses were indistinguishable in the mutant background ($p > 0.05$). The decremented responding of the mec-4 mutant could therefore not be facilitated by tap. In
summary, sensory input detected by the touch cells can dishabituate the ASH avoidance circuit.

Recently, Cho & Sternberg (2014) demonstrated that anterior or posterior body touch hastens the slowed reaction time associated with ASH activation during quiescence. Calcium imaging suggested that touch facilitated responding by increasing synchrony in the command interneurons. Although I found that input from body touch cells did not affect habituated reaction times, it did facilitate reversal duration. Promoting synchrony among command interneurons may explain how tap facilitates reversal duration in the dishabituation assay, especially if habituation training disrupts coordinated activity, as occurs during quiescence (Cho & Sternberg, 2014).

2.3.4 Dopamine signaling slows habituation

As described in chapter 1, the presence of food has been found to slow the decrement in response probability following repeated or prolonged exposure to CuCl$_2$, a water-soluble repellent detected by ASH (Ezcurra et al., 2011). Calcium transients in ASH matched the behavioral data, with a more rapid decline in cellular responding in the absence of food. Our habituation assay allowed us to test whether food was also modulating the avoidance circuit downstream of cell excitation, as ChR2 photocurrents bypassed the native transduction machinery. Comparing the same behavioral metric as Ezcurra et al. (2011; i.e. response probability), I found that populations tested in the absence of food were less likely to respond to the final stimulus than populations tested on a thin bacterial lawn, $t(10) = 3.2, p = 0.009$ (Fig.
However, initial responses were not affected by feeding state in this assay, $t(10) = 0.9, p = 0.38$. Although all other experiments in this chapter were conducted in the presence of a bacterial lawn, it is important to note that the food+ condition for Fig. 2.6A referred to a thin, nearly invisible bacterial lawn that had been spread with liquid culture at most 2 hours before testing (as in Ezcurra et al., 2011), in contrast with Fig. 2.1 or Figure 2.6B, C, and D, where animals were tested directly on their rearing plate with a much thicker bacterial lawn. The fact that repeated photoactivation recapitulated the rapid decrement in responding off of food that had been observed by Ezcurra et al. (2011) demonstrated that food was not only promoting responses upstream, but also downstream of ASH excitation.

Receptors for serotonin (SER-1, SER-5, and MOD-1), dopamine (DOP-3 and DOP-4), octopamine (OCTR-1, SER-3, and SER-6), and tyramine (TYRA-3) have all been found to alter naïve responding to a variety of ASH-sensed stimuli as a function of feeding state (Harris et al., 2010; Harris et al., 2009; Ezak and Ferkey, 2010; Ezcurra et al., 2011; Mills et al., 2011). The molecular underpinning of this phenomenon is complex, involving neuromodulators operating at multiple levels in the circuit (Harris et al., 2009; Komuniecki et al., 2012). To assess the role of monoamine neurotransmitters in the modulation of habituation, the ASHp::ChR2 transgene was crossed into strains with a mutation in $tph-1$, $cat-2$, or $tdc-1$, which encode tryptophan hydroxylase, tyrosine hydroxylase, and tyrosine decarboxylase, enzymes required for the biosynthesis of serotonin, dopamine, and tyramine/octopamine, respectively (Lints & Emmons, 1999; Sze et al., 2000; Alkema et al., 2005). In terms of the probability of responding to the final stimulus, a one-
way ANOVA showed a significant difference among the four strains, $F(3,20) = 15.6$, $p = 0.0001$. Tukey’s HSD comparisons indicated that the cat-2 mutant had a decreased likelihood of responding at the end of the assay ($p < 0.05$), while tph-1 and tdc-1 mutants were indistinguishable from control ($p > 0.05$; Fig. 2.6B). The serotonin-and dopamine-deficient mutants actually had opposite phenotypes, as habituation training diminished the importance of serotonin in the aversion response: the tph-1 mutant was more likely to respond to the final stimulus than to the first, $t(10) = 4.0$, $p = 0.003$. Loss of both dopamine and serotonin in a cat-4; bas-1 double mutant (Loer & Kenyon, 1993) resulted in a rapid habituation phenotype that was similar to the dopamine deficient cat-2 mutant (Fig. 2.6C) and reminiscent of animals tested in the absence of food (Fig. 2.6A). Although the likelihood of responding to the final stimulus was decreased in the cat-4; bas-1 double mutant compared to control, $t(10) = 6.9$, $p < 0.0001$, initial response probability was unaffected by the simultaneous loss of dopamine and serotonin, $t(10) = 0.5$, $p = 0.63$. Together these data suggest that dopamine signaling promoted responding to repeated ASH activation, while an appropriate serotonin/dopamine balance was essential for a robust naïve response.

*C. elegans* has eight dopaminergic neurons in three classes, CEP, ADE, and PDE. The texture of the bacterial food source is thought to activate these dopaminergic neurons directly requiring the mechanosensitive TRPN channel, TRP-4 (Li et al., 2006; Kindt et al., 2007). The trp-4 mutant, like the dopamine deficient cat-2 mutant, habituated more quickly than control to repeated photoactivation of ASH (Fig. 2.6D). In addition to the dopaminergic neurons, TRP-4 is expressed in at least 2 classes of interneurons, including DVA, where it functions as a stretch
receptor for proprioceptive feedback (Li et al., 2006). To better localize site of action, the *trp-4* mutant was compared with control and a strain expressing *trp-4* cDNA exclusively in dopaminergic neurons. Evaluating the probability of responding to the final stimulus, a one-way ANOVA showed a significant difference among the three strains, *F*(2,21) = 8.0, *p* = 0.0027. Tukey’s HSD comparisons indicated that the *trp-4* mutant had a decreased likelihood of responding at the end of the assay (*p* < 0.05), while the *trp-4* mutant with TRP-4 restored to dopamine neurons was indistinguishable from control (*p* > 0.05; Fig. 2.6D). Thus, the texture of the bacterial food source stimulates dopamine release, which promotes responding (i.e. slows habituation) to persistent aversive stimuli by modulating the avoidance circuit downstream of ASH excitation.

### 2.3.5 DOP-4 slows habituation

To identify the receptor through which food and dopamine slowed habituation, I evaluated loss-of-function phenotypes for known (*dop-1*, *dop-2*, *dop-3*, and *dop-4*) and potential (*dop-5* and *dop-6*) dopamine GPCRs. All mutants tested were indistinguishable from control animals for initial responding probability and only loss of DOP-4, an invertebrate-specific D1-like dopamine receptor (Sugiura et al., 2005), recapitulated the rapid habituation phenotype of the dopamine-deficient *cat-2* mutant (Fig. 2.7A). To confirm it as the causative mutation and to implicate ASH as the site of action, genomic *dop-4* was reintroduced to the *dop-4* mutant under control of its own or a *gpa-13* promoter. A one-way ANOVA showed a significant difference among strains for the probability of responding to the final
stimulus, $F(3,23) = 18.2, p < 0.00001$. Tukey’s HSD comparisons indicated that the dop-4 mutant with expression restored by the dop-4 promoter was indistinguishable from control ($p > 0.05$) and more likely to respond than either the dop-4 mutant or the mutant expressing dop-4 by the gpa-13 promoter ($p < 0.05$). Monitoring behavior and calcium transients, Ezcurra et al. (2011) previously reported that DOP-4 functioned upstream of ASH excitability to increase naïve sensitivity of ASH to water-soluble repellents in the presence of food. DOP-4 therefore acts both in ASH to modulate naïve sensitivity as a function of feeding state and in downstream neurons to promote responding to repeated stimulation.

The very low number of animals responding in the dop-4 mutant background at the end of the assay made it difficult to assess habituation of response magnitude metrics, i.e. duration and latency. Loss of the DOP-4 receptor did however affect the locomotory behavior during the periods between stimuli. Examining the 3s window immediately preceding a stimulus, the proportion of time spent moving forward increased across the assay similarly to control (Fig. 2.8A), but the dop-4 mutant moved forward at a faster speed after the final stimulus ($t(257) = 2.4, p = 0.017$; Fig. 2.8B).

2.3.6 Habituation training promotes dispersal

The behavioral plasticity associated with repeated ASH activation (i.e. accelerating forward speed and inhibiting reversals) would be expected to promote dispersal. Indeed, analyzing 20s worm track trajectories I observed increased displacement over the duration of the assay (Fig. 2.9). Consistent with its decreased
likelihood of reversing to ASH activation and elevated forward speed, the *dop-4* mutant travelled even further than control in the final 20s of the assay (*t* (185) = 7.5, *p* < 0.00001).

2.4 Discussion

I established a new *C. elegans* learning assay combining optogenetics to simulate naturally sensed stimuli with real-time computer vision software for highly detailed behavioral analysis. Repeated photoactivation of ASH had several effects on *C. elegans* behavior, including slowing reaction time and decreasing response duration. To test that a behavioral decrement qualifies as habituation, it is essential to rule out other potential causes, like adaptation, fatigue, or injury. In this case I also needed to confirm that our ectopic sensory transduction machinery, i.e. ChR2, was not adapting with repeated activation. This was supported by my behavioral data showing that repeated photoactivation decremented responding to a naturally sensed stimulus (i.e. nose touch; Fig. 2.4). Furthermore, dishabituating mechanosensory input could facilitate the decrement (Fig. 2.5), confirming it qualified as habituation. There are several advantages of this habituation assay. Firstly, by using ChR2 photocurrents to activate ASH, the sensory transduction machinery of ASH was bypassed, therefore preventing adaptation at this level and allowing us to probe downstream mechanisms of behavioral plasticity. Secondly, I specifically activated ASH and could therefore rule out a contribution from secondary sensory neurons known to contribute to avoidance behavior. Finally, I was able to quantify multiple behavioral metrics of a population receiving a
consistent and discrete stimulus. Habituation studies typically probe a single response metric, but as I show here, behaviors are rarely defined by one dimension.

Earlier work using naturally occurring cues demonstrated that persistent exposure to ASH-sensed stimuli results in a decreased likelihood of behavioral responding (Hart et al., 1999; Hilliard et al., 2005). Monitoring calcium currents in ASH, Hilliard et al. (2005) showed that the decrease in behavioral response correlates with a decrease in cellular responsiveness, to CuCl$_2$ at least, and that the behavioral and cellular decrement are dependent on GPC-1, a G$_\gamma$ also involved in behavioral adaptation to soluble attractants (Jansen et al., 2002). Decreased cellular responsiveness to specific compounds suggests that ASH can adapt to aversive stimuli (Hilliard et al., 2005; Hart et al., 1999). Adaptation at the level of stimulus detection can occur in parallel with more downstream mechanisms of habituation. Consistent with this concept, previous assays have reported much more rapid decrements in the probability of responding than those observed here, where I prevented adaptation by bypassing sensory transduction. Furthermore, loss of *gpc-1* or *osm-9* caused no obvious phenotypes in my optogenetic-based assay (data not shown), despite being implicated in ASH adaptation (Hilliard et al., 2005; Lindy et al., 2014). By deconstructing the behavioral response, I demonstrated explicitly that an ASH-mediated avoidance response could habituate.

Dopamine signaling promoted responding during habituation training and I identified the invertebrate specific D1-like dopamine receptor, DOP-4, as a key mediator. Dopamine is a neuromodulator frequently implicated in neural and behavioral plasticity, a role for dopamine signaling has also been proposed for
habituation in mammals (Lloyd et al., 2014). Consistent with this model, mesolimbocortical and nigrostriatal dopaminergic neurons respond to unconditioned salient and arousing sensory input (Horvitz, 2000). In C. elegans, dopamine modulates a variety of behaviors, including locomotion (Sawin et al., 2000; Hills et al., 2004; Vidal-Gadea et al., 2011) and learning (Kimura et al., 2010; Voglis & Tavernarakis, 2008; Hukema et al., 2008; Sanyal et al., 2004; Bettinger & McIntiire, 2004). Kindt et al. (2007) worked out the cellular pathway by which dopamine signaling attenuates habituation of the tap withdrawal response in the presence of food – mechanosensory dopaminergic neurons respond to the texture of the bacterial lawn and DOP-1 functions in the body touch receptor neurons to promote cell excitability by intracellular calcium release and PKC activity downstream of a Gq/PLC-β cascade. Ezcurra et al. (2011) also found that dopamine signaling slows the response decrement following persistent exposure to a water-soluble repellent, CuCl₂, however they did not observe a dop-4 phenotype. DOP-4 signaling may modulate habituation to some, but not all ASH-sensed stimuli. Alternatively, a habituation phenotype for the dop-4 mutant may have been masked by sensory adaptation to CuCl₂. Our optogenetic approach removes the contribution from adaption to specifically evaluate habituation, which may better reveal the dop-4 phenotype.

DOP-4 plays a role in the transition from thrashing-like swimming in liquid to crawling on agar (Vidal-Gadea et al., 2011), a qualitatively distinct gait in which animals maintain an S-shaped posture with deeper and slower dorsal-ventral bends (Pierce-Shimomura et al., 2008). It is possible that the circuitry underlying this
transition also drives ASH-mediated responding, as the elicited reversal can be considered a gait transition from forward to backward crawling. Although DOP-4 was not required for the naïve reversal response, habituation training induced an altered behavioral state in which dopamine, as opposed to serotonin, promoted responding. As with the naïve response to ASH activation, the opposite gait transition (crawl to swim) was dependent on a serotonin-dopamine balance. Dopamine plays an evolutionarily conserved role in motor pattern selection and in humans, loss of dopaminergic neurons causes the motor symptoms characteristic of Parkinson’s disease. Future work will need to define the site of DOP-4 function for both habituation and the swim to crawl gait transition.

Failure to avoid certain stimuli detected by ASH could be fatal for *C. elegans*. Why then do reversal responses habituate? My data show that different behavioral components show unique patterns of plasticity following repeated ASH activation, with habituation being just part of a strategy to promote dispersal from a dangerous locale. However, the avoidance circuit must balance this more long-term goal, with the short-term goal of evading noxious stimuli. The increased response latency associated with habituation may be a strategy to strike a balance between these long and short-term goals. Given the graded connection between ASH and reversal command interneurons (Lindsay et al., 2011), loss of an early immediate reversal response would allow animals to ignore less intense stimuli, like nose touch, while maintaining sensitivity to more serious threats, like osmotic shock. By promoting reversal responses, DOP-4 signaling prioritizes evasion over dispersal in the presence of a bacterial food source. The dual-process theory states that stimulation
induces both local circuit habituation and an organism-wide state of sensitization (Groves & Thompson, 1970). Sensitization may well be reflected in the shift in locomotion pattern induced by habituation training. A persistent aversive stimulus elicits the optimal escape strategy – minimize non-essential backward movement and accelerate forward.
Figure 2.1. Plasticity of reversal responses elicited by repeated ChR2 activation in ASH. (A) Representative raster plots depicting the behavioral state of 50 animals at the beginning (left) and end (right) of training. Pixels are color coded for velocity with negative values corresponding to backward locomotion. Black bars indicate 2s of whole-plate illumination with blue light at 250μW/mm². (B-D) Three different response metrics for reversals elicited by thirty 2s light pulses administered at 0.1Hz. White circles show the median, box limits indicate the 25th and 75th percentile, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, and polygons represent density estimates of the data (n=48 plates, 25±0.5 animals tracked per plate). Dotted line denotes median response to stimulus 1. All data from the ASHp::ChR2 transgenic background.
Figure 2.2. Shift in foraging behaviors during the interstimulus intervals. (A) Proportion of the population’s time spent moving forward, backward, or not at all during the 3s interval immediately preceding each stimulus delivered at 0.1Hz. (B) Speed of worms moving forward during the same 3s intervals. Mean +/- SEM. n=6 plates with 23±1.6 and 20±0.9 animals tracked per plate for ATR+ and ATR- conditions, respectively. All data from the ASHp::ChR2 transgenic background.
Figure 2.3. Spontaneous recovery from training with thirty 2s light pulses delivered at 0.1Hz. (A,B) Reversal duration and latency responses to a 2s light pulse at one of 4 recovery time points following training. Circles are plate means (24.5±0.6 animals tracked per plate) crosses are population means +/- SEM. The dash-dot line is the naïve response level and the dashed line is the habituated level. #, & and ¥ denote statistically distinguishable groups. (C) Above: Representative raster plots depicting the behavioral state of 50 animals for 10s at early (left) and late (right) recovery. Pixels are color coded for speed with negative values corresponding to backward locomotion. Below: Distribution of foraging behaviors following training (n=6 plates with 24.0±1.2 animals tracked per plate). (D) Forward speed deceleration following habituation training (n=6 plates with 24.0±1.2 animals tracked per plate). Dashed line is pre-training speed. All data from the ASHp::ChR2 transgenic background.
Figure 2.4. Generalization of natural stimuli and those simulated with optogenetics. (A) Nose touch responses were inhibited by training (stim = 2s light pulse x 20 at 0.1Hz). The majority of naive worms crawled backwards after a head-on collision with an eyelash, but for worms reared on ATR, the likelihood of a nose touch response was significantly reduced by prior ASH photoactivation. # and & denote statistically distinguishable groups (n=41, 56, 46, 52 animals/group). (B) Pre-exposure to octanol (oct) decremented the duration and increased the latency of reversals elicited by ChR2 photocurrents, as compared with pre-exposure to the ethanol vehicle (veh). Circles are plate averages, crosses are population averages +/- SEM. Control and osm-9 had 36.3±5.3 and 27.1±4.0 animals tracked per plate, respectively. Asterisks and n.s. denote statistically distinguishable and indistinguishable comparisons, respectively. All data from the sra-6p::ChR2 transgenic background.
Figure 2.5. Sensory input from body touch receptors acts as a dishabituating cue. (A,B) A tap after training diminished the decrement in reversal duration without influencing response latency. The dash-dot line is the initial response level and the dashed line is the habituated response level. The asterisk denotes statistically distinguishable groups. 23.8±1.4 animals tracked per plate. (C) Duration of the reversal response elicited by the initial and final 2s light pulse of training and the response after a dishabituating tap. The decrement of a touch insensitive mec-4 mutant was not dishabituated by tap. Control and mec-4 groups had 37.1±1.8 and 29.5±1.4 animals tracked per plate, respectively. #, &, and ¥ denote statistically distinguishable groups. Circles are plate means, crosses are population means +/- SEM. Data in 2.5A,B from the ASHp::ChR2 transgenic background and data in 2.5C from the sra-6p::ChR2 transgenic background.
Figure 2.6. Dopamine signaling promotes responding during habituation training. (A) Comparing populations off of food with those tested on a very thin bacterial lawn (n=6 plates with 19.2±1.3 and 18.8±2.1 animals tracked per plate for food+ and food- conditions, respectively). (B,C) Loss of monoamine biosynthetic enzymes altered the probability of responding (n=6 plates). For (B), the number of animals tracked per plate was 19.5±1.3, 20.3±1.3, 23.8±1, and 23±2 for +, tph-1, cat-2, and tdc-1, respectively. For (C), the number of animals tracked per plate was 20.3±2.1, 21±1.4, and 22±2.2 for +, cat-2, and bas-1;cat-4, respectively. (D) Loss of trp-4 recapitulated the dopamine deficient cat-2 mutant phenotype and could be rescued by expression in dopaminergic neurons (n=8 plates). Mean +/- SEM. # and & denote statistically distinguishable groups based on the likelihood of responding to the final stimulus. All data from the ASHp::ChR2 transgenic background.
Figure 2.7. Loss of *dop-4* recapitulates the phenotype of a dopamine deficient *cat-2* mutant. (A) Proportion of the population responding relative to control to the initial (top) and 30th (bottom) 2s light pulse delivered at 0.1Hz. The number of animals tracked per plate was 50±2, 58.8±4.6, 62.8±3.1, 27.8±1, 55±1.2, 19.8±2.6, 24.3±1.2, and 55.8±2.2 for *cat-2, dop-1*(vs101), *dop-1*(ok398), *dop-2, dop-3, dop-4, dop-5, and *dop-6*, respectively. Representative raster plots depicting control and *dop-4* responses to the initial (top) and final (bottom) stimulus. Pixels are color coded for speed with negative values corresponding to backward locomotion. Black bars indicate 2s of whole-plate illumination with blue light at 70μW/mm². (B) Proportion of animals responding to each stimulus of habituation training, with reintroduction of *dop-4* (*dop-4p::dop-4*) rescuing the rapid response decrement, but not if expression is restricted to ASH (*gpa-13p::dop-4*). Mean +/- SEM. # and & denote
statistically distinguishable groups based on the likelihood of responding to the final stimulus. Data from the sra-6p::ChR2 transgenic background, except for dop-2 and dop-5 mutants in the ASHp::ChR2 background.
Figure 2.8. Locomotory behavior of the dop-4 mutant. (A) Proportion of the population's time spent moving forward during the 3s interval immediately preceding each stimulus delivered at 0.1Hz. (B) Speed of worms moving forward during the same 3s intervals. Mean +/- SEM. # and & denote statistically distinguishable groups based on the final datapoint. n=4 plates for each strain with 61±2.4 and 19.8±2.6 animals tracked per plate for + and dop-4, respectively. All data from the sra-6p::ChR2 transgenic background.
Figure 2.9. Habituation training promotes dispersal. (A) Representative trajectories of 50 animals during the initial (left) and final (right) 20s of habituation training. Tracks were randomly assigned colors and start points were set to center. (B) Displacement (shortest distance between the start and endpoint) over 20s intervals during habituation training. Mean +/- SEM. # and & denote statistically distinguishable groups based on the final datapoint. n=4 plates for each strain with 61±2.4 and 19.8±2.6 animals tracked per plate for + and dop-4, respectively. All data from the sra-6p::ChR2 transgenic background.
3. Role of PDF signaling in habituation

3.1 Introduction

In chapter 2, I described the validation of a high-throughput habituation assay for ASH-mediated responses and how it was used to explore the attenuation of habituation in the presence of food via dopamine signaling through DOP-4. In this chapter, I identify a neuropeptide receptor and its ligands that promote dispersal and habituation of response magnitude.

Neuropeptides play key roles in a wide variety of processes and their importance in learning and memory is an emerging trend. It is predicted that the *C. elegans* genome has 119 neuropeptide precursor genes that can be processed into over 250 peptides (Li & Kim, 2008). In addition to the insulin/IGF receptor tyrosine kinases encoded by *daf*-2, there are over 100 predicted neuropeptide G protein-coupled receptors, the majority of which remain orphaned and functionally uncharacterized (Frooninckx et al., 2012). Insulin signaling has an especially prominent role in *C. elegans* behavioral plasticity (Chen et al., 2013; Lin et al., 2010; Vellai et al., 2006; Tomioka et al., 2006; Kodama et al., 2006), likely because of the use of feeding state as an unconditioned stimulus in associative learning paradigms. However, non-associative habituation assays have also been shown to depend on neuropeptide signaling. For example, prolonged exposure to a volatile attractant detected by AWC results in a decreased attraction to that odorant that can be reversed with a dishabituating stimulus (Colbert & Bargmann, 1995; Pereira & van der Kooy, 2012). Chalasani et al. (2010) found that the decreased attractiveness was dependent on NLP-1, a buccalin-related peptide expressed in AWC. Based on the
expression pattern of orphan neuropeptide GPCRs they managed to link NLP-1 with the NPR-11 GPCR using mutant analysis followed by biochemical confirmation. Expressing nlp-1 in AWC sensory neurons and npr-11 in AIA interneurons rescued the habituation deficits associated with each mutant. They propose a neuropeptide feedback loop, whereby NLP-1 released from the AWC sensory neurons acts on AIA to induce release of insulin-like peptide INS-1, which signals back to AWC to modulate odor sensitivity. In mechanosensory habituation, worms tapped 80 times at a 60s interval can remember training for at least 12h (Li et al., 2013). It is hypothesized that repeated taps recruit dense-core vesicles to the synaptic terminals of the body touch cells, leading to increased release of a neuropeptide, FLP-20 and smaller reversal responses. Short-term tap habituation may also be dependent on neuropeptide signaling, based on the slowed habituation phenotype of a neuropeptide synthesis mutant (egl-21) that was one of the 522 strains tested as part of Dr. Andrew Giles’ PhD thesis.

In addition to their role in learning, neuropeptides have also proven to be important modulators of ASH-mediated responding. As described in chapter 1, for octanol avoidance, NLP-3 peptides promote responding in the presence of food (Harris et al., 2010), while NLP-6, NLP-7, NLP-8, and NLP-9 peptides inhibit responses in the presence of octopamine (Mills et al., 2012). The importance of neuropeptides in the modulation of ASH avoidance responses was first appreciated by Kass et al. (2001), who in a suppressor screen for glr-1 nose touch insensitivity identified an allele of egl-3, which encodes a proprotein convertase required for the synthesis of neuropeptides (Gomez-Saladin et al., 1994; Husson et al., 2006).
Suppression of glr-1 phenotypes could be rescued by restoring egl-3 expression in a subset of interneurons downstream of ASH, including the AVA, AVD, and AVE reversal command interneurons (Kass et al., 2001). Inspired by this result, Mellem et al. (2002) demonstrated that loss of egl-3 also suppressed the increased response latency for glr-1-independent responding to osmotic shock. They also showed that the egl-3 suppression of glr-1 phenotypes is absolutely dependent on NMDA glutamate receptor subunit, NMR-1 and that glutamate currents in AVA and AVD are unaffected by the loss of egl-3. Based on these findings they proposed that loss of neuropeptides caused an increased concentration of synaptic glutamate, which activated extrasynaptic NMDA receptors. However, the key neuropeptides were never identified.

Using the assay described in chapter 2, I too identified glr-1 phenotypes that could be suppressed by an egl-3 allele. To identify the relevant neuropeptide signal(s), I conducted a targeted RNAi suppressor screen against known and potential neuropeptide GPCRs. Based on this screen I implicated pigment dispersing factor (PDF) neuropeptide signaling in habituation of ASH-mediated responses.

3.2 Methods

3.2.1 Strains

The ChR2 transgenes were crossed into FX04393, KP1580, LSC27, MT1241, MT6308, TU3595, VC671, VC2609, VM3109, and VM3804 to generate the following strains:
VG186 lin-15b(n744); sid-1(pk3321) yvls1; ulS72[unc-119p::sid-1 + mec-18p::GFP + myo-2p::mCherry]

VG222 egl-21(n611); lite-1(ce314) ljls114
VG223 glr-1(ky176); lite-1(ce314) ljls114
VG227 nmr-1(ak4); lite-1(ce314) ljls114
VG232 eat-4(ky5); lite-1(ce314) ljls114
VG234 egl-3(ok979); lite-1(ce314) ljls114
VG244 egl-3(ok979); glr-1(ky176); lite-1(ce314) ljls114
VG250 glr-1(ky176); GLR-1::GFP; lite-1(ce314) ljls114
VG264 pdfr-1(ok3425); lite-1(ce314) ljls114
VG380 pdf-2(tm4393); yvls1
VG382 pdf-1(tm1996); yvls1
VG383 pdfr-1(ok3425); yvls1
VG393 pdf-2(tm4393); pdf-1(tm1996); yvls1

For cAMP overexpression in pdfr-1 positive neurons, pSF180 [pdfr-1p::ACY-1(P260S)-sl2-mCherry (50ng/ul)] was injected into the gonad of the pdf-1;pdf-2 double mutant VG393. For pdfr-1 rescue experiments, pSF134 [pdfr-1p::inv[pdfr-1.d-sl2-GFP] (35ng/ul)] was co-injected with one of several nCre expressing plasmids (1ng/ul) for cell-specific Cre-lox recombination in pdfr-1 mutant VG383. pSF180, pSF134, and two of the seven nCre expressing plasmids (pSF11 [tag-168p::nCre] & pSF176 [eat-4p::nCre]) were gifts from Cori Bargmann (The Rockefeller University) and are described in more detail in Flavell et al. (2013). Also co-injected was pCFJ90
(myo-2p::mCherry (2ng/ul); Frøkjær-Jensen et al., 2008) for use as a visible marker and pBluescript to make the total DNA concentration 100ng/ul (Mello et al., 1991).

The following strains were generated by microinjection:

VG411, VG412 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + glr-1p::nCre + myo-2p::mCherry]

VG434, VG438, VG441 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + *eat-4p::nCre* + myo-2p::mCherry]

VG442, VG443, VG446 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + *npr-1p::nCre* + myo-2p::mCherry]

VG447, VG448, VG449 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + *tag-168p::nCre* + myo-2p::mCherry]

VG481, VG82, VG483, VG484 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + *gcy-36p::nCre* + myo-2p::mCherry]

VG485, VG486, VG487, VG488 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + *myo-3p::nCre* + myo-2p::mCherry]

VG492 *pdf-1*(tm1996); *pdf-2*(tm4393); yvls1; yvEx152[ *pdfr-1p::ACY-1*(P260S)-sl2-mCherry + myo-2p::mCherry]

VG507, VG508, VG509, VG510 *pdfr-1*(ok3425); yvls1; yvEx[ *pdfr-1*::inv[ *pdfr-1.d-sl2-GFP*] + *ocr-4p::nCre* + myo-2p::mCherry]

3.2.2 Plasmid construction

To generate the nCre expression vectors, promoters were subcloned into plasmid pSF11 [ *tag-168p::nCre*], cut with FseI and Ascl.
A 2kb *myo-3* promoter was amplified from plasmid KP#1866 (a gift from Josh Kaplan, Harvard University) using oligos 5’-
CTTAACGGCCGGCCTGTGTGTGATTGCTTTTTCACAATC-3’ and 5’-
ACACTTGCGCCTCTAGATGGATCTAGTGGTGTTG-3’.

A 2.7kb *glr-1* promoter was amplified from plasmid pSH128 (a gift from Alexander Gottschalk, Goethe University Frankfurt) using oligos 5’-
CTTAACGGCCGGCCTTTCAAGTGTCCTGTTGTC-3’ and
5’-ACACTTGCGGCGCTGTGAATGTGTCAGATTGG-3’.

A 3.4kb *npr-1* promoter was amplified from N2 genomic DNA using oligos
5’-CTTAACGGGCACCGAAACGCAGTTGGCAACAAG-3’ and
5’-ACACTTGCGCCTCTGCTATGTCTGAAATTT-3’.

A 1.1kb *gcy-36* promoter was amplified from N2 genomic DNA using oligos
5’-CTTAACGGGCGCCATGATTTGGTAGATGGGT-TTGG-3’ and
5’-ACACTTGCGCCTTTGAGCCCTTGTGGGATTT-3’.

A 4.8kb *ocr-4* promoter was amplified from N2 genomic DNA using oligos
5’-CTTAACGGGCCTTAAAGACCTTGGCTCCAC-3’ and
5’-ACACTTGCGGCGCTTAATACAAAGGTAGATTGAGAGA-3’.

3.2.3 RNAi

Systemic RNAi was performed essentially as described previously (Kamath et al., 2001; Kamath et al., 2003). Briefly, RNAi plates were composed of NGM agar, 1 mM IPTG, and 5 µM ATR seeded with overnight liquid culture of *E. coli* strain HT115 carrying either control plasmid L4440 or an RNAi vector targeting *egl-3* or one of 57
GPCRs. One or two days after seeding, VG186 adults were bleached onto the RNAi plates and the first generation adults were tested behaviorally.

3.3 Results
3.3.1 glr-1 phenotypes

Of the known stimuli detected by ASH, only the nose touch response appears to be absolutely dependent on the AMPA glutamate receptor subunit and GluR1 homolog, GLR-1 (Hart et al., 1995; Maricq et al., 1995). Although they do not respond to nose touch, animals lacking glr-1 are able to initiate backward locomotion to osmotic shock, albeit with an increased latency (Mellem et al., 2002). It is hypothesized that the ASH output evoked by a nose touch is insufficient to elicit a response in animals lacking GLR-1 function, but that other glutamate receptors are recruited for more intense stimuli, like osmotic shock. Using the optogenetic-based habituation assay developed in chapter 2, I tested glutamate transmission mutants lacking the AMPA receptor subunit, GLR-1, the NMDA receptor subunit, NMR-1, or the glutamate vesicular transporter, EAT-4 (Fig. 3.1). In terms of the probability of responding to the initial stimulus, a one-way ANOVA showed a significant difference among strains, $F(3,20) = 28.1$, $p < 0.00001$. Tukey’s HSD comparisons indicated that the eat-4 mutant had a decreased likelihood of responding ($p < 0.05$), while glr-1 and nmr-1 mutants were indistinguishable from control ($p > 0.05$). Although the likelihood of responding was unaffected by the loss of nmr-1 or glr-1, a one-way ANOVA showed a significant difference among these strains for reaction time, $F(2,239) = 8.2$, $p = 0.0004$. Tukey’s HSD comparisons indicated that the glr-1 mutant
was slower to respond than either the control or the nmr-1 mutant \( p < 0.05 \); Fig. 3.1B). In terms of the final stimulus, a one-way ANOVA again showed a significant difference among strains for the probability of responding, \( F(3,20) = 69.2, p < 0.00001 \). Tukey's HSD comparisons indicated that at the end of the assay, the control animals were more likely to respond than either the eat-4 or glr-1 mutant \( p < 0.05 \); Fig. 3.1C). The phenotypes associated with the glr-1 mutant could be at least partially rescued by expressing a GLR-1::GFP transgene from the glr-1 promoter – compared to the mutant, the rescue strain was faster to respond to the initial stimulus \( t(179) = 5.8, p < 0.00001 \); Fig 3.2A) and more likely to respond to the final stimulus \( t(8) = 4.2, p = 0.003 \); 3.2A; Fig 3.2B). In summary, glr-1 mutant animals expressing ChR2 in ASH initially reversed to a 2s blue light pulse with a slowed reaction time, however this glr-1-independent response did not persist with repeated stimulation. Loss of the glutamate vesicular transporter, EAT-4, dramatically impaired the probability of a reversal response, while loss of NMDA receptor subunit NMR-1 had no discernable effect. In naïve animals other glutamate receptor subunits can compensate for the loss of GLR-1, but with repeated stimulation, reversals require this major excitatory receptor subunit. Based on these results, I hypothesized that ASH habituation was associated with decreased glutamatergic signaling.

3.3.2 Loss of egl-3 suppresses glr-1

Blocking neuropeptide synthesis with a mutant allele of egl-3 had previously been shown to suppress the glr-1 phenotype associated with nose touch and
osmotic shock (Kass et al., 2001; Mellem et al., 2002). Consistent with these results, I found that the glr-1; egl-3 double mutant responded more quickly to initial ASH photoactivation than the glr-1 single mutant \((t(128) = 3.8, p = 0.0002; 3.2A)\), and was more likely to respond to the final stimulus \((t(8) = 4.5, p = 0.002; \text{ Fig. 3.2C})\). Like control animals, the egl-3 single mutant maintained a high probability of responding across the trial, however loss of egl-3 did impair habituation of response latency, as the mutant had a faster reaction time than control to the final stimulus \((t(211) = 5.9, p < 0.00001; \text{ Fig. 3.2D})\). To confirm that this response latency habituation phenotype could be attributed to a loss of neuropeptide signaling, the ChR2 transgene was crossed into a strain with a mutation in the gene encoding EGL-21, a major carboxypeptidase required for the synthesis of most neuropeptides (Jacob & Kaplan, 2003; Husson et al., 2007). In terms of the probability of responding, a one-way ANOVA showed no significant difference between strains for the initial stimulus, \(F(2,15) = 0.8, p = 0.48\), and although a difference was detected for the final stimulus, \(F(2,15) = 4.1, p = 0.04\), a Tukey’s HSD comparison indicated that neither the egl-3 nor egl-21 mutant differed from control \((p > 0.05; \text{ Fig. 3.3A})\). However, the neuropeptide synthesis mutants did have a deficit in response latency habituation, as a one-way ANOVA found the strains to be indistinguishable for the initial stimulus, \(F(2,244) = 0.6, p = 0.54\), but not for the final stimulus \(F(2,298) = 11.6, p = 0.00001\). A Tukey’s HSD comparison indicated faster reaction times at the end of the assay for both mutant strains \((p < 0.05; \text{ Fig. 3.3B})\). In summary, loss of egl-3 or egl-21 did not affect the probability of responding across the assay, but the reaction time of the mutants did not slow to the same degree as control with repeated
stimulation, suggesting that habituation of ASH-mediated reversals is mediated by neuropeptide signaling.

3.3.3 GPCR RNAi suppressor screen

To identify the neuropeptide signal responsible for the egl-3 and egl-21 habituation phenotypes, I used systemic RNAi to knockdown known and potential neuropeptide receptors in a glr-1 mutant background. The nervous system of *C. elegans* is generally refractory to RNAi by feeding, but can be sensitized by neuron-specific expression of the dsRNA channel, SID-1, in a *lin-15b* mutant background (Calixto et al., 2010). While the previous experiments of this chapter used a ChR2 transgene dependent on FLP Recombinase to specifically target expression to ASH (Ezcurra et al., 2011), a more responsive strain with the sra-6p::ChR2 transgene was used for the RNAi screen. The sra-6 promoter expresses strongly in ASH and more faintly in a pair of sensory neurons (ASI) and interneurons (PVQ; Troemel et al., 1995). The pattern of behavioral plasticity in this strain is the same as the strain with ChR2 expression restricted to ASH (as discussed in chapter 2). As had been seen with the mutant allele, RNAi knockdown of egl-3 suppressed the slowed reaction time of the naïve glr-1 mutant ($t(139) = 5.7, p < 0.00001$; Fig 3.4A), as well as its decreased probability of responding to the final ($t(4) = 6.9, p = 0.002$; Fig. 3.4B). I evaluated the loss of function phenotype for 57 GPCRs. In figures 3.4C and 3.4D the RNAi clones are ranked by the degree to which they decreased response latency to the initial stimulus and increased the probability of responding to the final stimulus, respectively. Knocking down egl-3 had the biggest effect for both
measures and, after correcting for multiple comparisons (values corresponding to |z-scores| > 4.46 were considered distinct from the control distribution; p < (0.05/57 =) 0.0008, two-tailed), I identified one other clone for each measure that was statistically distinguishable from the control. Knockdown of npr-26 reduced naïve response latency (Fig 3.4A,C), while knockdown of pdfr-1 resulted in an increased likelihood of responding at the end of the assay (Fig 3.4B,D). Table 3.1 lists the z-scores of all RNAi clones for three different metrics for the first and last stimulus of the assay, i.e. response probability, latency, and duration. Because my focus was in understanding the plasticity of the behavior, I followed up on the role of pdfr-1 in habituation of ASH-mediated responses.

3.3.4 PDF signaling mediates habituation

The PDFR-1 receptor has three known ligands encoded by two precursor genes, pdf-1 (encodes PDF-1a and PDF-1b) and pdf-2 (encodes PDF-2; Janssen et al., 2008). Using available loss of function alleles, I tested pdfr-1, pdf-1, and pdf-2 single mutants, as well as a pdf-1;pdf-2 double mutant. Evaluating the magnitude of the response to the final stimulus, a one-way ANOVA showed a significant difference among the strains, both in terms of reversal duration (F(4,806) = 10.5, p < 0.00001) and latency (F(4,806) = 22.5, p < 0.00001). For both response metrics, Tukey’s HSD comparisons indicated reduced habituation relative to control for both the pdf-1;pdf-2 double mutant and the pdfr-1 single mutant (p < 0.05), but not for the pdf-1 or pdf-2 single mutant (p > 0.05; Fig. 3.5A,B). Importantly, the habituation deficit cannot be attributed to differences in naïve responding, as Tukey’s HSD
comparisons indicated that the initial response of both the pdf-1;pdf-2 double mutant and the pdfr-1 single mutant is indistinguishable from control for both response metrics ($p > 0.05$). These data suggest that the PDF ligands function redundantly via PDFR-1 to promote habituation of response magnitude.

As is typical for secretin-like receptors, PDFR-1 is thought to signal through Gαs to stimulate cAMP synthesis from ATP. Indeed, HEK239 cells expressing PDFR-1 show a dose-dependent increase in cAMP levels with PDFR-1 activation (Janssen et al., 2008). Furthermore, Flavell et al. (2013) were able to ameliorate a pdfr-1 mutant locomotion phenotype by stimulating cAMP synthesis in pdfr-1 expressing neurons. In an attempt to associate the pdf-1; pdf-2 mutant habituation phenotype with loss of PDFR-1 signaling, the pdfr-1 promoter was used to drive expression of a constitutively active adenylyl cyclase (ACY-1(P260S); Saifee et al., 2011; Flavell et al., 2013), which catalyzes the conversion of ATP to cAMP and pyrophosphate. Comparing the initial response of control, the pdf-1; pdf-2 double mutant, and the double mutant expressing ACY-1(P260S), a one-way ANOVA showed no significant difference among the strains, both in terms of reversal duration ($F(2,198) = 2.6, p = 0.07$) and latency ($F(2,198) = 0.9, p = 0.42$). Therefore, chronic elevation of cAMP in pdfr-1 expressing cells did not simply induce a habituated state. Comparing the final responses, a one-way ANOVA showed a significant difference among the strains for reversal latency ($F(2,208) = 12.1, p = 0.00001; \text{Fig } 3.5C$), but not duration ($F(2,208) = 2.7, p = 0.07; \text{Fig } 3.5D$). Considering only response latency, Tukey’s HSD comparisons indicated that the strain with constitutively active ACY-1 was indistinguishable from control ($p > 0.05$), with both initiating responses more
slowly than the pdf-1;pdf-2 double mutant ($p < 0.05$). Stimulating cAMP synthesis in the appropriate cells could therefore compensate for loss of the PDF ligands, suggesting PDF signaling is permissive (rather than instructive) for habituation of ASH-mediated responses.

The mutant analysis of PDF signaling was done using the control strain with the sra-6p::ChR2 transgene, which causes off-target expression of ChR2 in ASI and PVQ. However, ASI and PVQ express PDF-1 and PDFR-1, respectively (Janssen et al., 2009; Barrios et al., 2012). The potential implications of off-target ChR2 expression was evaluated with the ASH-specific ChR2 transgene (ASHp::ChR2; Ezcurra et al., 2011). I found that the habituation phenotypes associated with pdfr-1 were still apparent when ChR2 expression was restricted to ASH (Fig. 3.6) – for the final stimulus, the mutant responded more quickly than control ($t(165) = 2.4, p = 0.02$) with a longer-lasting reversal ($t(165) = 2.8, p = 0.005$). An essential role for ASI and PVQ involvement was thereby ruled out.

3.3.5 PDFR-1 functions in neurons and muscle

PDFR-1 is expressed in all body wall muscle cells, as well as in neurons in the head and tail (Janssen et al., 2008; Barrios et al., 2012). In an attempt to identify where PDFR-1 functions to promote habituation, I used the intersectional promoter rescuing strategy (and several reagents) described by Flavell et al. (2013), in which different Cre recombinase expression vectors are co-injected with inverted and floxed pdfr-1 cDNA driven by a pdfr-1 promoter. Full rescue was defined as scores distinct from the mutant and statistically indistinguishable from the control and
partial rescue was defined as scores distinct from both mutant and control. Pan-neuronal expression of Cre (tag-168 promoter) rescued the response latency phenotype in 3 out of 3 lines tested (Fig 3.7A). In contrast, restoring pdfr-1 expression in the body wall muscle (myo-3 promoter) only partially rescued the response latency phenotype in 1 out of 4 lines (Fig 3.7C). Despite having normal habituation of response latency, reversal duration was not rescued in any of the 3 strains with pan-neuronal Cre expression (Fig. 3.7B). However, 1 of the 4 muscle expression lines displayed a partial rescue (Fig. 3.7D). Reversal duration habituation may be more sensitive to cAMP concentration in neurons or require PDFR-1 signaling in both neurons and muscle. These data demonstrate that the latency and duration response metrics are dissociable, even if mediated by the same molecular component, i.e. pdfr-1. To identify key neurons for response latency habituation, 5 different Cre expression plasmids (npr-1p::Cre, glr-1p::Cre, eat-4p::Cre, gcy-36p::Cre, and ocr-4p::Cre) with partially overlapping expression patterns were used to restore PDFR-1 expression to subsets of cells, as summarized in Table 3.2. Lines expressing Cre by the npr-1 and eat-4 promoters partially rescued the response latency habituation phenotype (Fig 3.7C). These promoters intersect with each other and pdfr-1 in PQR, PHA, and PHB. PQR and the other two npr-1 and pdfr-1 intersecting cell classes (URX and OLQ) were targeted for Cre-expression with gcy-36 and ocr-4 promoters, as were two of the other eat-4 and pdfr-1 intersecting cell classes (PVQ and URY) with the glr-1 promoter. However, none had any effect on response latency habituation (Fig 3.7C), making PHA and/or PHB the most likely cells for PDFR-1 signaling to promote habituation. Their involvement will need to be
confirmed using a rescue strain with more restricted PDFR-1 expression. PHA and PHB are sensory neurons of the phasmid sensilla in the worm’s tail. Previous work demonstrated that at least one chemical repellent (SDS) detected by ASH also activates PHA and/or PHB (Hilliard et al., 2002). PHA/PHB neurons inhibit reversals elicited by ASH, thereby allowing integration of sensory input from the head and tail to prevent animals from backing into aversive compounds. PHA/PHB may be similarly stimulated by PDFR-1 signaling to inhibit responding to persistent ASH activation. Other neurons are likely involved, as pan-neuronal Cre expression fully rescued the behavioral deficit that could only be partially rescued by Cre expression with npr-1 or eat-4 promoters.

3.3.6 PDF signaling promotes dispersal to persistent sensory input

Previous mutant analyses suggest that PDF signaling promotes roaming over dwelling behavior in both hermaphrodites and males (Meelkop et al., 2012; Flavell et al., 2013; Barrios et al., 2013). This behavior has been attributed to sensory, motor, and interneurons, as well as muscle (Flavell et al., 2013; Barrios et al., 2013; Choi et al., 2013). I measured basal locomotion by quantifying displacement over the 30s window immediately preceding the habituation assay for pdfr-1, pdf-1, and pdf-2 single mutants, as well as the pdf-1;pdf-2 double mutant. A one-way ANOVA showed a significant difference among strains ($F(4,750)=39.9$, $p < 0.00001$). Tukey’s HSD comparisons revealed three distinguishable levels, control populations travelled further than all other strains ($p < 0.05$), while the pdf-1 and pdf-2 single mutants travelled further than the double mutant or the pdfr-1 single mutant ($p < 0.05$; Fig.
Therefore, consistent with previously described locomotion deficits (Meelkop et al., 2012; Flavell et al., 2013), I found that the PDF ligands functioned partially redundantly to promote displacement via the PDFR-1 receptor. Evaluating the pdfr-1 rescue lines described above, pdfr-1 expression in neurons completely rescued the spontaneous locomotion phenotype, while expression in muscle partially rescued it (Fig. 3.8C). I also observed partial rescue with lines in which npr-1, glr-1, or eat-4 promoters drove Cre (Fig. 3.8C). These three promoters do not intersect in any one class of neuron, suggesting a distributed circuit for PDFR-1 function. Rescuing basal locomotion without affecting habituation (as in the line expressing Cre by the glr-1 promoter) rules out locomotion deficits as the cause of the habituation phenotype.

In chapter 2, I demonstrated that repeated ASH activation promoted dispersal behavior. As discussed above, PDF promotes dispersal during spontaneous locomotion. I evaluated dispersal behavior during habituation training by measuring displacement over the first and final 30s of the assay. Within-strain comparisons revealed an increase in control animals, \( t(267) = 13.7, p < 0.00001 \), that was not apparent for either the pdfr-1 mutant, \( t(344) = 0.3, p = 0.75 \), or the pdf-1;pdf-2 double mutant, \( t(169) = 0.6, p = 0.56 \) (Fig. 3.9A,B). The pdfr-1 phenotype was also apparent with the ASH-specific ChR2 transgene, as displacement was higher at the end versus the start of the assay for the control, \( t(190) = 6.1, p < 0.00001 \), while the pdfr-1 mutant exhibited the opposite trend (Fig. 3.10A). Evaluating the pdfr-1 rescue lines described above, I found that the dispersal increase could be mediated by PDFR-1 signaling in neurons or muscle (Fig 3.9C).
Restoring PDFR-1 expression in muscle most reliably rescued the deficit in stimulation-induced dispersal. The results for each rescue line for each metric can be found in Table 3.3.

Light entrainment of circadian rhythms in Drosophila is dependent on PDF signaling (Renn et al., 1999). Thus, illumination as a stimulus was a potentially important confound of my assay. Although wild-type controls or retinal negative ChR2 transgenic worms did not reverse to the light stimulus, I could not rule out illumination in combination with repeated ASH activation initiating a PDF signaling cascade to promote dispersal. I therefore tested the habituation of a behavior that could be elicited without the use of light, the tap withdrawal response. I demonstrated for the first time that repeated taps also promoted dispersal, as the displacement of the control population was higher over the final 30s of the assay than during the first 30s, \( t(163) = 5.8, p < 0.00001 \). This behavioral change was dependent on PDFR-1, as the pdfr-1 mutant displayed the opposite trend (Fig. 3.10B). Persistent sensory activity therefore promotes dispersal via PDF signaling in at least two neural circuits.

3.4 Discussion

Phenotypes associated with neuropeptide synthesis mutants, egl-3 and egl-21, inspired me to perform a targeted RNAi screen to identify the key neuropeptides and their receptors. This screen led me to PDFR-1 and its ligands, encoded by pdf-1 and pdf-2, which were essential for normal habituation of ASH-mediated responses. PDF signaling is highly conserved across the protostomian evolutionary lineage.
First discovered in crustaceans, the Pigment-Dispersing-Hormones were named for their role in regulating the daily cycles of color change (Rao & Riehm, 1993). The PDF receptors are distantly related to mammalian calcitonin GPCRs and vasoactive intestinal peptide receptors. The mammalian vasoactive intestinal peptide receptor and Drosophila PDF receptor have conserved function in the regulation of arousal and circadian rhythms (Kunst et al., 2014). Janssen et al. (2008) provided the first functional characterization of PDF signaling in C. elegans. They reported that loss of pdf-1, the precursor gene that encodes ligands PDF-1a and PDF-1b, resulted in slow moving animals with elevated rates of spontaneous reversals and directional changes, a deficit mimicked by overexpression of the other PDF neuropeptide, encoded by precursor pdf-2. Consistent with antagonistic action of the ligands, Meelkop et al. (2012) found that loss of pdf-2 suppressed the pdf-1 mutant's locomotion phenotype. However, Flavell et al. (2013) found that the pdf-2 mutation exasperated it, a relationship I also observed with the displacement metric. This is indicative of partially redundant ligands.

The PDF gene is expressed in sensory, inter- and motor neurons (Janssen et al., 2009), but it remains to be determined which are releasing PDF ligands to promote habituation of ASH-mediated reversals. Of particular interest are those neurons co-expressing pdf-1 and pdf-2 (AIM, PHA, PHB, PQR, PVP, PVT, RID) and those with which ASH has direct synaptic output (ADA, ASK, AVB, AVD, PVP, RIM, RMG). PVP is the only neuron class that meets both criteria. In terms of site of action, PDFR-1 signaling in neurons appears essential for robust habituation of response latency, but not response duration – with the phasmid neurons PHA
and/or PHB being the best candidates (Fig. 3.7). Consistent with previous reports, I identified a more distributed PDFR-1 circuit regulating spontaneous locomotion (Fig 3.8C; Flavell et al., 2013; Barrios et al., 2013; Choi et al., 2013), while signaling in neurons and muscle appears to promote dispersal with repeated ASH activation (Table 3.3).

In *Drosophila* PDF signaling is a key regulator of the circadian cycle via rhythmic release from a small group of central clock cells, the ventral lateral neurons, to promote arousal during waking (Renn et al., 1999; Kunst et al., 2014). Although *C. elegans* displays circadian rhythms in locomotion and abiotic stress resistance (Saigusa et al., 2002; Kippert et al., 2002), the underlying mechanism is unclear. *C. elegans* homologues of the *Drosophila* clock genes are thought to control developmental timing, e.g. *lin-42* (*period*), *tim-1* (*timeless*), and *kin-20* (*doubletime*; Jeon et al., 1999; Banerjee et al., 2005). Choi et al. (2013) showed that secretion of PDF-1 neuropeptides is developmentally regulated. During the lethargus period that precedes larval molting, less PDF-1::YFP fusion proteins accumulate in the scavenger cells of the body cavity. Reduced PDF-1 secretion is thought to underlie the behavioral quiescence associated with this period, as it could be blocked by forced PDF-1 release. In adulthood, PDF also signals arousal, promoting roaming over dwelling behavior, with apparently spontaneous switching between these states occurring every several minutes (Flavell et al., 2013). It is unclear what internal drives mediate the abrupt transitions during spontaneous locomotion, but my data suggest that the roaming state associated with PDF signaling can also be induced by persistent sensory input, i.e. repeated activation of ASH.
In summary, habituation training induces a roaming state by PDFR-1 signaling in muscle and neurons, while signaling in neurons mediates habituation of response latency. Other neuropeptides are likely involved, as loss of PDFR-1 did not totally abrogate habituation, as was seen with neuropeptide synthesis mutants *egl-3* and *egl-21*. Persistent sensory input as arousing stimuli is not unique to the ASH avoidance circuit, as tap habituation also promotes PDFR-1 dependent dispersal.
Figure 3.1. Naive response and habituation phenotypes for glutamate transmission mutants. (A) Representative raster plots depicting the behavioral state at the beginning (left) and end (right) of training. Pixels are color coded for speed with negative values corresponding to backward locomotion. Black bars indicate 2s of whole-plate illumination with blue light at 250μW/mm². (B) Time to initiate a reversal from the onset of the first stimulus in a habituation series. Circles are plate means, crosses are population means +/- SEM. (C) Proportion of the population reversing to each of 30 two-second light pulses administered at 0.1Hz. The glr-1 independent response did not persist across the assay. Mean +/- SEM. The number of animals tracked per plate was 24±1.7, 23.5±1.4, 23.3±1.7, and 11.2±1.8 for +, glr-1, nmr-1, and eat-4, respectively. # and & denote statistically distinguishable groups.
based on the likelihood of responding to the final stimulus. All data from the ASHp::ChR2 transgenic background.
Figure 3.2. The *glr-1* phenotypes can be suppressed by expressing GLR-1 or knocking out *egl-3*. (A) Time to initiate a reversal from the onset of the first stimulus in a habituation series. Circles are plate means, crosses are population means +/- SEM. #, &, and ¥ denote statistically distinguishable groups. (B and C) Proportion of the population reversing to each of 30 two second light pulses administered at 0.1Hz. # and & denote statistically distinguishable groups based on the likelihood of responding to the final stimulus. (D) Time to initiate a reversal from the onset of each stimulus. Mean +/- SEM. The number of animals tracked per plate was 29±1.3, 28±1.5, 29.4±1.2, 16±1.3, and 16.4±0.9 for +, *glr-1, glr-1;GLR-1::GFP, glr-1;egl-3*, and *egl-3*, respectively. #, &, and ¥ denote statistically distinguishable groups based on the reaction time to the final stimulus. All data from the ASHp::ChR2 transgenic background.
Figure 3.3. Neuropeptide synthesis mutants do not display habituation of response latency. (A) Proportion of the population reversing to each of 30 two second light pulses administered at 0.1Hz. (B) Time to initiate a reversal from the onset of each stimulus. # and & denote statistically distinguishable groups based on the reaction time to the final stimulus. Mean +/- SEM. n=4 plates for each strain with 29.3±1.7, 12.8±1.9, and 14.7±1.7 animals tracked per plate for +, egl-3, and egl-21, respectively. All data from the ASHp::ChR2 transgenic background.
Figure 3.4. GPCR RNAi screen. (A) Time to initiate a reversal from the onset of the first stimulus and (B) proportion of animals reversing to the final stimulus for populations fed RNAi clones to knock down *egl-3* or one of 57 GPCRs. Each circle is the mean of 3 plates, with multiple replicates for the control and *egl-3* targeting vector. Dashed lines mark upper and lower critical values (i.e. |z-score| = 4.46). (C) Time to initiate a reversal to the initial stimulus for animals fed the RNAi clones that decreased reaction time. Circles are plate means, crosses are population means +/- SEM. #, &, and ¥ denote statistically distinguishable groups. (D) Proportion of the population reversing to each stimulus for animals fed the RNAi clones that increased the probability of a reversal to the final stimulus. Mean +/- SEM. # and & denote statistically distinguishable groups based on the likelihood of responding to the final stimulus. All data from the *sra-6p::ChR2* transgenic background.
Figure 3.5. PDF signaling promotes habituation. (A and C) Time to initiate a reversal from the onset of each of 30 two second light pulses administered at 0.1Hz. (B & D) Reversal duration for each stimulus. Mean +/- SEM. # and & denote statistically distinguishable groups based on the response to the final stimulus. n= 4 (A & B) or 3 (C & D) plates per strain. For (A & B) the number of animals tracked per plate was 71±8.1, 44±3.3, 42.8±4.3, 32.8±1.3, and 21.8±3.6 for +, pdfr-1, pdf-1, pdf-2, and pdf-1;pdf-2, respectively. All data from the sra-6p::ChR2 transgenic background.
Figure 3.6. *pdfr-1* mutant phenotype in control strain with ChR2 expression restricted to ASH. (A) Time to initiate a reversal from the onset of each of 30 two-second light pulses administered at 0.1Hz. (B) Reversal duration for each stimulus. Mean +/- SEM. # and & denote statistically distinguishable groups based on the reaction time to the final stimulus. n=6 plates per strain with 22.5±1 and 13±1.7 animals tracked per plate for + and *pdfr-1*, respectively. All data from the ASHp::ChR2 transgenic background.
Figure 3.7. Restoring pdfr-1 expression can rescue the mutant habituation phenotypes. (A) Time to initiate a reversal and (B) reversal duration to each stimulus for pdfr-1 mutants with expression restored to neurons (pdfr-1;Ex[tag-168p::Cre + pdfr-1p::inv[pdfr-1]]) compared to control (+) or the pdfr-1 mutant (pdfr-1). Mean +/- SEM. n = 2-3 plates. # and & denote statistically distinguishable groups based on the response to the final stimulus. Degree to which each Cre expression plasmid rescued (C) response latency and (D) reversal duration habituation phenotypes of the pdfr-1 mutant coinjected with inverted and floxed pdfr-1 expression.
pdf\textit{r-1} cDNA. Mean response to the final five stimuli normalized with 1 as control and 0 as mutant for animals tested on the same day. Circles are plate means, crosses are population means +/- SEM. & and ¥ denote complete and partial rescue, respectively. 2-4 lines were tested for each Cre expression plasmid. Where applicable, data from a rescuing line is presented. All data from the \textit{sra-6p::ChR2} transgenic background.
Figure 3.8. PDF signaling promotes dispersal during spontaneous locomotion. (A) Representative trajectories of 50 animals during the 30s immediately preceding the habituation assay. Tracks were randomly assigned colors and start points were set to center. (B) Displacement (shortest distance between the start and endpoint) during the same period. Circles are plate means, crosses are population means +/- SEM. The number of animals tracked per plate was 71±8.1, 44±3.3, 42.8±4.3, 32.8±1.3, and 21.8±3.6 for +, pdfr-1, pdf-1, pdf-2, and pdfr-1;pdf-2, respectively. #, & and ¥ denote statistically distinguishable groups. (C) Degree to which each Cre expression plasmid rescued the spontaneous dispersal phenotype of the pdfr-1 mutant coinjected with inverted and floxed pdfr-1 cDNA. Displacement over the 30s immediately preceding the habituation assay normalized with 1 as control and 0 as mutant responses for animals tested on the same day. Circles are plate means, crosses are population means +/- SEM. & and ¥ denote complete and partial rescue, respectively. 2-4 lines were tested for each Cre expression plasmid. Where applicable, data from a rescuing line is presented. All data from the sra-6p::ChR2 transgenic background.
Figure 3.9. PDF signaling promotes dispersal during habituation training. (A) Representative trajectories during a 20s interval at the beginning (0-20s), middle (140-160s), and end (280-300s) of the habituation assay with 30 two second light pulses delivered at 0.1Hz. Tracks were randomly assigned colors and start points were set to center. (B) Displacement (shortest distance between the start and endpoint) over the first (left) and last (right) 30s of the assay for the PDF signaling mutants. The number of animals tracked per plate was 71±8.1, 44±3.3, 42.8±4.3, 32.8±1.3, and 21.8±3.6 for +, pdfr-1, pdf-1, pdf-2, and pdf-1;pdf-2, respectively. (C) Displacement over the first (left) and last (right) 30s of the assay for the pdfr-1 mutant rescue lines. 2-4 lines were tested for each Cre expression plasmid. Where applicable, data from a rescuing line is presented. # denotes a significant increase in
displacement (one-tailed, $p < 0.05$, with Bonferroni correction for multiple comparisons) over the assay. Circles are plate means, crosses are population means +/- SEM. All data from the sra-6p::ChR2 transgenic background.
Figure 3.10. Persistent sensory input promotes dispersal. Displacement (shortest distance between the start and endpoint) over the first (left) and last (right) 30s of an assay with repeated (A) ASH-specific photoactivation (22.5±1 and 13±1.7 animals tracked per plate for + and pdfr-1, respectively) and (B) repeated plate taps (42±3.2 and 36±7.9 animals tracked per plate for + and pdfr-1, respectively). Circles are plate means, crosses are population means +/− SEM. # denotes a significant increase. All data from the ASHp::ChR2 transgenic background.
Table 3.1. GPCR loss of function phenotypes for three behavioral metrics for the initial and final stimulus reported as Z-scores based on the control distribution.

<table>
<thead>
<tr>
<th>Geneservice location</th>
<th>Target</th>
<th>Proportion reversing</th>
<th>Reversal latency</th>
<th>Reversal duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>initial   final</td>
<td>initial   final</td>
<td>initial   final</td>
</tr>
<tr>
<td>I-3C12</td>
<td>ckr-1</td>
<td>0.70      -2.53</td>
<td>0.16      -1.99</td>
<td>1.47      0.79</td>
</tr>
<tr>
<td>I-3M14</td>
<td>nmur-4</td>
<td>0.57      -2.55</td>
<td>-0.59     -0.29</td>
<td>0.42      1.09</td>
</tr>
<tr>
<td>I-6K10</td>
<td>ntr-1</td>
<td>0.91      -2.87</td>
<td>0.50      -1.65</td>
<td>0.11      -0.77</td>
</tr>
<tr>
<td>II-1G01</td>
<td>nmur-2</td>
<td>1.50      -1.07</td>
<td>-3.83     1.66</td>
<td>0.10      -0.23</td>
</tr>
<tr>
<td>II-1G18</td>
<td>frpr-14</td>
<td>0.59      -2.18</td>
<td>-1.78     0.22</td>
<td>-0.23     1.67</td>
</tr>
<tr>
<td>II-6M20</td>
<td>npr-20</td>
<td>0.04      -2.49</td>
<td>1.12      2.83</td>
<td>-0.48     -0.92</td>
</tr>
<tr>
<td>II-8P03</td>
<td>dmsr-3</td>
<td>1.62      -2.06</td>
<td>-3.74     1.69</td>
<td>1.50      0.49</td>
</tr>
<tr>
<td>II-9C14</td>
<td>npr-34</td>
<td>0.56      -3.59</td>
<td>1.43      1.00</td>
<td>3.08      2.10</td>
</tr>
<tr>
<td>II-9K23</td>
<td>dmsr-6</td>
<td>-0.26     -2.20</td>
<td>-0.28     0.09</td>
<td>-1.44     0.18</td>
</tr>
<tr>
<td>III-1B07</td>
<td>ckr-2</td>
<td>-0.15     0.49</td>
<td>-2.06     -0.54</td>
<td>1.52      0.36</td>
</tr>
<tr>
<td>III-1H14</td>
<td>npr-15</td>
<td>3.45      -1.08</td>
<td>-1.91     -1.78</td>
<td>0.36      0.63</td>
</tr>
<tr>
<td>III-3D23</td>
<td>pdfr-1</td>
<td>-1.96     4.59</td>
<td>1.01      1.81</td>
<td>-0.55     0.60</td>
</tr>
<tr>
<td>III-4H24</td>
<td>F59B2.13</td>
<td>0.48     -0.44</td>
<td>-1.02     0.01</td>
<td>-0.06     1.41</td>
</tr>
<tr>
<td>III-4N22</td>
<td>npr-29</td>
<td>0.15      -0.10</td>
<td>-0.22     -1.05</td>
<td>-1.29     -0.55</td>
</tr>
<tr>
<td>III-5C01</td>
<td>tkr-1</td>
<td>-0.74     -0.64</td>
<td>0.28      0.45</td>
<td>0.91      2.10</td>
</tr>
<tr>
<td>III-5H08</td>
<td>dmsr-5</td>
<td>-0.23     -1.20</td>
<td>-0.32     1.42</td>
<td>-0.20     0.36</td>
</tr>
<tr>
<td>IV-2L21</td>
<td>tkr-3</td>
<td>-1.62     -2.99</td>
<td>0.88      3.19</td>
<td>-0.01     -0.26</td>
</tr>
<tr>
<td>IV-3K17</td>
<td>tkr-2</td>
<td>0.34      -2.95</td>
<td>-2.06     2.13</td>
<td>-0.09     2.19</td>
</tr>
<tr>
<td>IV-3L02</td>
<td>npr-35</td>
<td>0.08      -2.79</td>
<td>0.80      2.09</td>
<td>-0.39     -0.83</td>
</tr>
<tr>
<td>IV-3L09</td>
<td>npr-2</td>
<td>0.84      0.16</td>
<td>-2.62     -2.50</td>
<td>0.25      0.45</td>
</tr>
<tr>
<td>IV-4P04</td>
<td>npr-27</td>
<td>1.04      0.00</td>
<td>-1.58     -0.46</td>
<td>-1.28     0.82</td>
</tr>
<tr>
<td>IV-6I19</td>
<td>npr-3</td>
<td>1.14      -2.35</td>
<td>-1.53     -0.01</td>
<td>1.13      -0.50</td>
</tr>
<tr>
<td>IV-7G21</td>
<td>srsx-25</td>
<td>1.33      0.54</td>
<td>0.11      -0.82</td>
<td>-0.16     1.50</td>
</tr>
<tr>
<td>IV-8G18</td>
<td>npr-26</td>
<td>0.86      -0.23</td>
<td>-4.67     0.53</td>
<td>0.80      0.54</td>
</tr>
<tr>
<td>IV-8M21</td>
<td>npr-32</td>
<td>-0.04     -2.68</td>
<td>0.32      0.25</td>
<td>0.64      0.06</td>
</tr>
<tr>
<td>V-1G19</td>
<td>T22H9.1</td>
<td>1.81    -0.04</td>
<td>0.96      0.47</td>
<td>0.03      0.33</td>
</tr>
<tr>
<td>V-4P14</td>
<td>dmsr-12</td>
<td>0.71      0.30</td>
<td>0.05      0.00</td>
<td>-0.30     0.93</td>
</tr>
<tr>
<td>V-5A11</td>
<td>dmsr-14</td>
<td>-1.25     -1.04</td>
<td>-0.56     0.04</td>
<td>-0.80     -0.71</td>
</tr>
<tr>
<td>V-5A13</td>
<td>dmsr-13</td>
<td>0.85      0.14</td>
<td>-2.93     0.17</td>
<td>-0.31     1.35</td>
</tr>
<tr>
<td>V-5C05</td>
<td>frpr-18</td>
<td>0.52      0.17</td>
<td>-1.09     -1.88</td>
<td>0.66      0.07</td>
</tr>
<tr>
<td>V-5G08</td>
<td>srsx-24</td>
<td>0.07      0.39</td>
<td>-1.02     -1.54</td>
<td>-1.23     1.37</td>
</tr>
<tr>
<td>V-5L13</td>
<td>frpr-3</td>
<td>1.32      1.54</td>
<td>-3.00     -2.11</td>
<td>-1.31     -0.16</td>
</tr>
<tr>
<td>V-6006</td>
<td>frpr-6</td>
<td>2.03      1.83</td>
<td>-1.24     -0.92</td>
<td>-1.32     1.18</td>
</tr>
<tr>
<td>V-7B04</td>
<td>npr-25</td>
<td>1.44      -0.07</td>
<td>-3.94     -0.40</td>
<td>1.28      0.81</td>
</tr>
<tr>
<td>V-7D16</td>
<td>dmsr-1</td>
<td>0.74      0.35</td>
<td>-1.86     -0.30</td>
<td>-0.25     -0.30</td>
</tr>
<tr>
<td>V-7D24</td>
<td>T11F9.1</td>
<td>1.71      2.18</td>
<td>-0.31     -0.98</td>
<td>-1.04     0.63</td>
</tr>
<tr>
<td>Geneservice location</td>
<td>Target</td>
<td>Proportion reversing</td>
<td>Reversal latency</td>
<td>Reversal duration</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------</td>
<td>----------------------</td>
<td>------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>initial</td>
<td>final</td>
<td>initial</td>
</tr>
<tr>
<td>V-7G01</td>
<td>egl-3</td>
<td>-0.02</td>
<td>5.99</td>
<td>-8.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.70</td>
<td>5.76</td>
<td>-10.86</td>
</tr>
<tr>
<td>V-8N12</td>
<td>frpr-5</td>
<td>-0.05</td>
<td>-0.22</td>
<td>-0.13</td>
</tr>
<tr>
<td>V-8O10</td>
<td>frpr-15</td>
<td>0.34</td>
<td>-0.97</td>
<td>-0.58</td>
</tr>
<tr>
<td>X-1K03</td>
<td>npr-19</td>
<td>0.43</td>
<td>0.10</td>
<td>-0.97</td>
</tr>
<tr>
<td>X-2E06</td>
<td>npr-16</td>
<td>0.58</td>
<td>-2.83</td>
<td>-0.89</td>
</tr>
<tr>
<td>X-2F18</td>
<td>npr-1</td>
<td>-1.13</td>
<td>-2.35</td>
<td>-0.20</td>
</tr>
<tr>
<td>X-2N15</td>
<td>nmur-3</td>
<td>-0.72</td>
<td>1.22</td>
<td>-0.94</td>
</tr>
<tr>
<td>X-3J02</td>
<td>npr-8</td>
<td>-0.23</td>
<td>-1.08</td>
<td>-1.34</td>
</tr>
<tr>
<td>X-4C24</td>
<td>npr-28</td>
<td>0.73</td>
<td>0.13</td>
<td>-2.13</td>
</tr>
<tr>
<td>X-4D19</td>
<td>nmur-1</td>
<td>-0.02</td>
<td>-0.68</td>
<td>0.77</td>
</tr>
<tr>
<td>X-4F14</td>
<td>npr-18</td>
<td>-0.71</td>
<td>-2.76</td>
<td>2.14</td>
</tr>
<tr>
<td>X-4M14</td>
<td>frpr-8</td>
<td>-0.60</td>
<td>0.17</td>
<td>0.79</td>
</tr>
<tr>
<td>X-4N13</td>
<td>npr-7</td>
<td>0.07</td>
<td>-0.85</td>
<td>0.02</td>
</tr>
<tr>
<td>X-5E05</td>
<td>npr-6</td>
<td>2.02</td>
<td>2.72</td>
<td>-0.27</td>
</tr>
<tr>
<td>X-5P24</td>
<td>npr-4</td>
<td>1.76</td>
<td>1.82</td>
<td>1.04</td>
</tr>
<tr>
<td>X-6I21</td>
<td>npr-10</td>
<td>0.38</td>
<td>0.14</td>
<td>1.41</td>
</tr>
<tr>
<td>X-6J14</td>
<td>sprr-2</td>
<td>0.43</td>
<td>3.07</td>
<td>0.11</td>
</tr>
<tr>
<td>X-7C08</td>
<td>npr-33</td>
<td>1.14</td>
<td>0.63</td>
<td>-0.89</td>
</tr>
<tr>
<td>X-7H14</td>
<td>npr-24</td>
<td>0.74</td>
<td>0.55</td>
<td>2.69</td>
</tr>
<tr>
<td>X-7J08</td>
<td>frpr-7</td>
<td>0.04</td>
<td>-1.25</td>
<td>1.98</td>
</tr>
<tr>
<td>X-7M11</td>
<td>F59D12.1</td>
<td>0.69</td>
<td>0.55</td>
<td>0.79</td>
</tr>
<tr>
<td>X-7P13</td>
<td>npr-21</td>
<td>0.96</td>
<td>0.27</td>
<td>-0.26</td>
</tr>
</tbody>
</table>
Table 3.2. Reported expression pattern of pdfr-1 and the 7 promoters used to drive Cre.

<table>
<thead>
<tr>
<th></th>
<th>pdfr-1</th>
<th>tog-168</th>
<th>grl-1</th>
<th>cad-4</th>
<th>npr-1</th>
<th>goy-36</th>
<th>ocr-4</th>
<th>myo-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLQ</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>URX</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PQR</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PHA</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PHB</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ALM</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AVM</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FLP</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>OLL</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PLM</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>URY</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PVQ</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AVD</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PVC</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RME</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Other neurons</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>x</td>
</tr>
</tbody>
</table>

Reference

Janssen et al., 2008; Barrios et al., 2012
Ishihara & Katsura, unpublished
Maricq et al., 1995; Hart et al., 1995
Serrano-Saiz et al., 2013
Coates & de Bono, 2002
Cheung et al., 2004
Tobin et al., 2002
Miller et al., 1983
Table 3.3. PDFR-1 rescue experiments summary.

<table>
<thead>
<tr>
<th>Cre promoter</th>
<th>Strain</th>
<th>Latency habituation</th>
<th>Duration habituation</th>
<th>Spontaneous dispersal</th>
<th>Habituated dispersal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VG447</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>tag-168</td>
<td>VG448</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG449</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG411</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG412</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG434</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>glr-1</td>
<td>VG438</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG441</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG442</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG443</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG446</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG481</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG482</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG483</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG484</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG507</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG508</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG509</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG510</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG507</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG508</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG509</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG510</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VG485</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG486</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG487</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VG488</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
4. Discussion

Habituation is a fundamental process poorly understood at the cellular and molecular levels. Taking advantage of the tractable nervous system of *C. elegans*, I have developed novel insights into habituation by investigating responses elicited by a pair of polymodal nociceptors, ASHL and ASHR. The ASH neuron class is especially interesting in the context of habituation because of the diversity and salience of cues it detects. The objectives of this research were:

1) To establish a high-throughput habituation assay for ASH-mediated responses.

2) To identify molecular components mediating behavioral plasticity.

In chapter 2, I demonstrated that repeated ASH photoactivation caused behavioral plasticity meeting two of the key characteristics for habituation set out by Spencer and Thompson (1966). Specifically, that “repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level” and “presentation of a different stimulus results in an increase of the decremented response to the original stimulus.” I found that repeated photoactivation caused shorter reversal responses that could be facilitated by a tap to the side of the Petri plate. Thus the decrement could not be explained by sensory adaptation or fatigue. Instead of focusing on just one parameter of the response, I characterized locomotion throughout training and identified a suite of behavioral changes associated with repeated ASH activation. Habituation is typically viewed as a way to free up limited cognitive resources, but my data highlights a different perspective, i.e. habituation as part of a shifting behavioral strategy, in this case to
promote dispersal. Towards objective 2, I identified two key signaling pathways that sculpted the behavioral plasticity. Food and dopamine promoted responding via DOP-4 (chapter 2), while PDF signaling promoted habituation of response latency and duration (chapter 3).

4.1 A new high-throughput habituation assay

Studying ASH-mediated reversals by way of the drop test, nose touch, or “stink-on-a-stick” single worm assays is labor intensive, furthermore the stimulus strength and timing is difficult to control. I adapted the Multi-Worm Tracker to accommodate optogenetic experiments, allowing for precise temporal and intensity regulation of a stimulus that could be administered to an entire population simultaneously. This greatly enhanced the reproducibility and throughput of the assay, with a significant gain in behavioral detail. In addition, by bypassing the native sensory transduction machinery, I prevented sensory adaptation and with combinatorial genetics could specifically target ASH without activating secondary sensory neurons known to contribute to avoidance of aversive stimuli. This was useful for evaluating habituation of ASH-mediated responses, but it also represents an important deviation from normal neural activity. The primary value of this assay lies in the gains in throughput and behavioral detail. Hypotheses generated with optogenetics should be validated using naturally sensed stimuli whenever possible. Caveats of the optogenetic approach are addressed in more detail below. There are two main issues: (1) that the control worms are not wild-type and (2) that the stimulus is simulated with light.
Optogenetic experiments require targeting light-sensitive proteins to cells that normally do not express them. Overexpression of any foreign protein can alter the structure, function, or survival of cells. However expression of ChR2 in ASH did not noticeably alter responses elicited by a naturally sensed stimulus, i.e. nose touch (Fig. 2.4A). Another concern is the mutations caused by integration of the transgenes. This was controlled for by the use of two strains in which the transgene for ChR2 expression integrated at different sites in the genome. The use of these two strains also supports the robustness of the results, as the strains differed in several ways. One strain expressed ChR2 strongly in ASH and weakly in two classes of off-target cells, while the other had ChR2 restricted to ASH. The ASH-specific strain had lower ChR2 expression levels and had to be illuminated with increased irradiance, which required a lite-1 mutant background (lite-1 encodes a native C. elegans short-wavelength light receptor; Edwards et al., 2008). Despite these differences, the two strains displayed the same patterns of behavior in the assays described and were similarly affected by the subset of mutant alleles tested in both, i.e. cat-2, glr-1, eat-4, egl-3, egl-21, and pdfr-1. Thus, the effects of repeated ASH photoactivation are consistent between genetic backgrounds, stimulus intensities, and ChR2 transgenes.

But how do they relate to naturally sensed stimuli?

Optogenetics has been used extensively to simulate natural neural activity, but it is important to consider the caveats associated with photoactivation. The first issue is the use of light itself, which could cause photodamage or even just alter neural activity by heating. Light could also be detected by native receptors. My LED system delivered very low intensity blue light (max 250 μW/mm²), but to control
for illumination I evaluated the behavior of wild-type as well as retinal-negative transgenic worms. Neither control displayed any appreciable behavioral response to repeated illumination. The second issue relates to the photocurrent itself, which deviates from native signal transduction in terms of kinetics, localization, and ion content. This may be especially relevant for neurons with graded transmission, like ASH, where the extent of depolarization is directly correlated with the photocurrent, as opposed to spiking neurons, which need only be depolarized to threshold before a digital action potential is generated by native channels. Finally, there are also differences at the circuit level, as secondary sensory neurons normally co-activated with ASH may play a role in decoding input. Furthermore, adaptation normally occurs in parallel with habituation and removing this component by bypassing sensory transduction could have significant impacts on behavioral plasticity. Thus a major limitation of this assay is the inability to assess the role of adaptation in habituation. Despite these caveats, the optogenetic approach recapitulated observations from experiments using naturally sensed cues. Most notably the dopamine-dependent slowed decrement in responding to persistent CuCl$_2$ in the presence of food (Ezcurra et al. 2011) and the suppression of the $glr$-$1$ mutant’s nose touch and osmoavoidance deficits by loss of $egl$-$3$ (Kass et al., 2001; Mellem et al., 2002). It is impossible to know how ASH photoactivation was perceived by the animals, but genetic analysis of glutamate transmission mutants suggested it was not experienced as a nose touch, as Hart et al. (1995) and Maricq et al. (1995) demonstrated that reversals elicited by nose touch depend on GLR-$1$. For osmotic shock, Mellem et al. (2002) observed that the $glr$-$1$ mutant responded to ASH
photoactivation with a slowed reaction time, as I observed here, suggesting the photostimulus was more akin to osmotic pressure. Taken together, the experiments reported here demonstrate that the use of optogenetics to simulate naturally sensed stimuli is a valid and useful model for studying habituation of ASH-mediated responses. It is worth noting that most habituation assays are only approximations of reality because of the use of unnaturally consistent and reliable stimuli.

As part of a collaborative side project, I also used an optogenetic approach to provide a mechanistic insight into habituation to tap (Timbers et al., 2013). We had observed tap habituation rates increasing throughout adulthood and hypothesized that this was caused by decreased sensitivity to mechanical stimuli in older animals, as one of the key characteristics of habituation is an inverse relationship between stimulus intensity and rate of decrement (characteristic #5; Spencer & Thompson, 1966; Rankin et al., 2009). To test this hypothesis, transduction machinery was bypassed using ChR2 activation in the body touch cells to simulate tap. As predicted, habituation to these simulated taps does not change across adulthood, suggesting that the aging effect is mediated by a decline in sensitivity upstream of cell excitation. This was confirmed by adjusting the force of the tap, which had a more profound effect on the habituation of younger animals. Behavioral analysis of many neural circuits could benefit from the gains in stimulus control, throughput, and quantification granted by the coupling of optogenetics and the Multi-Worm Tracker.
4.2 Similarities, differences, and interactions of converging circuits

I set out to study habituation of a behavioral response similar to the reversal elicited by plate taps. ASH activation promotes reversals that depend on circuitry partially overlapping with that mediating the tap withdrawal response. However ASH detects diverse and potentially lethal cues and I therefore anticipated some degree of divergence for habituation of the two circuits. Like ASH, activation of the body touch cells by tap elicits a backward crawling response that often ends in a directional change known as an omega turn. There is however differences at the behavioral level, for example reversals elicited by the body touch cells are typically larger and are also associated with a suppression of head oscillations that occurs with some, but not all ASH sensed stimuli (Alkema et al., 2005; Piggott et al., 2011). Although the reversal responses are mediated by a largely overlapping set of interneurons (most notably AVA, AVD, and RIM), the sensory cells signal to these synaptic partners differently. While glutamatergic transmission from ASH drives a reversal response, neurotransmitter release from the body touch cells primarily modulates the reversal mediated by gap junction coupling to the command interneurons (Hart et al., 1999; Mellem et al., 2002; Chalfie et al., 1985; Wicks & Rankin, 2000). Furthermore, the non-localized agar vibration evoked by tap activates both a forward and backward drive, the integration of which determines the behavioral outcome (Wicks & Rankin, 1995). I therefore expected some similarities and some differences with respect to habituation of these converging circuits.
The first immediately obvious difference is that compared with ASH photoactivation, the probability of a reversal declines more readily with repeated taps or with taps simulated via photoactivation of the body touch cells (Nagel et al., 2005; Timbers et al., 2013). However, using naturally sensed stimuli others have shown a rapid decline in the probability of responding to repeated or prolonged ASH activation (Hilliard et al., 2005; Ezcurra et al., 2011). This decrement is likely caused by decreased sensitivity to the stimulus (i.e. sensory adaptation) that cannot occur with photostimulation. Therefore, for the ASH avoidance circuit adaptation and habituation normally occur in parallel to primarily affect the probability and magnitude (respectively) of the response, whereas repeated taps causes habituation downstream of sensory transduction that decreases both the likelihood and magnitude of the response.

For both ASH and the body touch cells, the presence of food promotes responding to repeated stimulation through elevated dopamine levels (Kindt et al., 2007; Ezcurra et al., 2011). For tap habituation this is mediated by D1-like dopamine receptor DOP-1 attenuating the decrease in touch cell excitability to repeated stimulation (Kindt et al., 2007). While dopamine also slows the rate of response decrement to repeated CuCl₂ exposures in the presence of food, the relevant receptor had not been identified (Ezcurra et al., 2011). Using the high-throughput optogenetic assay described in chapter 2, I implicated the invertebrate-specific dopamine receptor DOP-4. The deficits of each dopamine receptor appear to be assay-specific, as loss of DOP-4 did not effect tap habituation (data not shown) and loss of DOP-1 did not affect habituation of the ASH avoidance circuit (Fig. 2.7).
The rapid habituation phenotype of the *dop-4* mutant could not be rescued by restoring expression in ASH, suggesting that unlike DOP-1, DOP-4 was not functioning in the sensory neuron itself. Although DOP-1 and DOP-4 are both thought to be D1-like receptors, DOP-4 belongs to an invertebrate specific subfamily, which includes the *Drosophila* receptor, DAMB, and the *Apis mellifera* receptor, DOP-2 (Sugiura et al., 2005). DOP-4 expression has been reported in ASG, AVL, CAN, and PQR, pharyngeal neurons I1 and I2, as well as vulva, intestine, rectal glands, and rectal epithelial cells (Sugiura et al., 2005). The site of DOP-4 function is still under investigation. Although habituation of both responses is modulated by food and dopamine, the key dopamine receptors and their site of action differ. Other organisms should be tested to determine if dopaminergic modulation of habituation is shared across phylogeny.

Knowledge gleaned from tap habituation studies should be evaluated with the ASH avoidance circuit and vice versa. For example, in the touch cells, autophosphorylation by the potassium channel accessory subunit MPS-1 promotes habituation (Cai et al., 2009). MPS-1 is also expressed in ASH, where it is thought to associate with K+ channel KVS-1 (Bianchi et al., 2003). As was done for tap (Cai et al., 2009), habituation of the ASH avoidance circuit should be tested with the *mps-1* mutant expressing a kinase dead variant. The other uncloned tap habituation mutants, i.e. *adp-1* and *hab-1* (Swierczek et al., 2011; Xu et al., 2002), should also be tested in this new assay. Downstream of DOP-1, Kindt et al. (2007) implicated a Gq/PLC-β signaling cascade by showing loss of EGL-30 and EGL-8 phenocopied the loss of DOP-1. A similar approach could be used to define the relevant pathway.
downstream of DOP-4, starting with a mutant analysis of Gα subunit orthologs for Gq (egl-30), Go (goa-1), and Gs (gsa-1). Conversely, findings originating from the ASH avoidance assay may be relevant for tap habituation. Indeed, I found that repeated taps also promoted dispersal and that this was dependent on PDFR-1 signaling (Fig. 3.10). The pdfr-1 rescue lines described in chapter 3 should be evaluated with repeated taps to compare sites of action. As demonstrated by DOP-1 and DOP-4, not all molecular components of habituation are predicted to generalize to all circuits and some are not great candidates, for example loss of glutamate vesicular transporter EAT-4 results in rapid tap habituation, but simply impairs naïve responding to all ASH-sensed stimuli (Hart et al., 1999; Mellem et al., 2002; Guo et al., 2009). The use of multiple habituation assays is necessary for testing the generalizability of mechanistic insights.

The successful use of tap to dishabituate ASH-mediated responses demonstrates an interaction between these two converging circuits. Interestingly, the opposite interaction does not occur, that is ASH photoactivation does not dishabituate the tap-withdrawal response (data not shown). Rather than reversing the habituation process, sensory input from the touch cells appears to facilitate ASH-mediated reversals by recruiting a sensitizing mechanism that gets superimposed on the decrement. The major piece of evidence for this is the inability for dishabituation to accelerate recovery to baseline, i.e. although a dishabituating plate tap facilitates decremented responses, the time to reach baseline is indistinguishable from spontaneously recovering controls (data not shown). Furthermore, in naïve animals tap facilitates responses above baseline with a
similar time course to dishabituation, approximately 1 min (data not shown). Evidence for dishabituation as a superimposed facilitatory process has also been reported in other systems, for example the cat leg flexion reflex (Spencer et al., 1966; Groves & Thompson 1970), the leech shortening reflex (Ehrlich et al. 1992; Sahley et al. 1994), and the Aplysia gill- and siphon-withdrawal reflexes (Carew et al. 1971; Cohen et al. 1997; Antonov et al. 1999; Hawkins et al., 2006). So far the mechanism underlying sensitization and dishabituation of ASH-mediated responses is unknown, as the behaviors were intact in all mutants tested to date, except touch insensitive mec-4 mutants. A potential mechanistic clue comes from Cho & Sternberg (2014), who demonstrated that body touch promotes coordinated interneuron activity to facilitate ASH-mediated responding during quiescence, a period of reduced responsivity (Raizen et al., 2008). A similar phenomenon may underlie dishabituation and sensitization.

4.3 Conclusion

Habituation is typically framed as a process allowing animals to ignore irrelevant stimuli in order to free up limited cognitive resources (Rankin et al., 2009). However, my data highlight the need for a broader interpretation. Careful behavioral analysis of an intact freely moving animal revealed that habituation could not be sufficiently described by the decrement of any single response metric, as it is so often reduced. Repeated stimulation actually induced a suite of behavioral changes that are at least partially genetically dissociable, but together define the state of the organism. For persistent ASH photoactivation, reversal responses are
largely maintained, but their duration shortens and reaction time slows, while the motile fraction of the population and its speed increases. Together these changes promote displacement, suggesting that in this case habituation is not so much about ignoring sensory input, as it is part of a shifting strategy prioritizing dispersal. Although this interpretation is not expected to generalize to every habituating circuit, my approach highlights the value of detailed behavioral analysis of an intact, freely moving animal.

By dissecting behavior into multiple metrics, I identified two pathways that shaped the plasticity - dopamine and PDF signaling. Dopamine promoting ASH-mediated responses is reminiscent of its role signaling salience in mammals (Horvitz, 2000), while the arousing influence of invertebrate PDF neuropeptides are mirrored by mammalian vasoactive intestinal peptide, whose receptors are similar in sequence to PDFR-1 (Kunst et al., 2015). Habituation is not a single process; rather, it comprises multiple mechanisms operating at different levels of neural circuits. While some mechanisms may be vertebrate-specific, others are more conserved. For example, the large conductance voltage- and calcium-activated potassium (BK) channel mediates short-term habituation in mice, flies, and worms (Engel & Wu, 1998; Typlt et al., 2013). The best chance for a complete characterization of mechanisms of habituation relies on the continued use of diverse genetic model organisms with tractable nervous systems.
References


