BEHAVIOURAL AND GENETIC DISSOCIATION OF FACILITATORY PROCESSES
IN *CAENORHABDITIS ELEGANS*

by

Alex Jian Tong Yu

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

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in Neuroscience

**Examining Committee:**

Catharine Rankin
Supervisor
Michael Gordon
Supervisory Committee Member

Supervisory Committee Member
Kurt Haas
Additional Examiner

**Additional Supervisory Committee Members:**

Stan Floresco
Supervisory Committee Member

Supervisory Committee Member
Abstract

The simplest forms of learning are non-associative learning. In non-associative learning, both sensitization and dishabituation cause an increase in the behavioural response. A strong and/or surprising stimulus can enhance a naïve response above the baseline in sensitization, or facilitate a previously decremented response in dishabituation. While a lot has been done to unravel the cellular and molecular mechanisms for sensitization, the mechanism(s) for dishabituation remains elusive. Sensitization and dishabituation were considered as the same facilitatory process for decades, but recent evidence suggests that they may be two separate processes. The focus of this thesis was to investigate whether sensitization and dishabituation are the same or two different processes. In this research, sensitization and dishabituation of the response to optogenetic stimulation of ASH nociceptor neurons by a mechanosensory tap stimulus were characterized and compared in a series of behavioural paradigms in C. elegans. It was found that sensitization and dishabituation were produced in four ASH response components with different time-dependent dynamics. Using a candidate gene approach, the effects of several genes on sensitization and dishabituation of the ASH response were examined. It was found that genes played differential roles in mediating the two facilitatory processes; genes that were critical for sensitization did not affect dishabituation. Taken together, these findings strongly suggest that sensitization and dishabituation are two facilitatory processes mediated by distinct genetic and molecular pathways. This research deepens our understanding of the complex mechanisms of “simple” forms of learning.
Lay Summary

Non-associative learning is classified as a simple form of learning, that is widely observed in virtually all animal species. Despite being a universal and fundamental form of learning, surprisingly little is known about the brain mechanisms to mediate non-associative learning. This research was dedicated to further our understanding of how the nervous system functions to support non-associative learning. The results of this study found that two forms of non-associative learning, both increasing the behaviour, can be produced by the same stimulus presented in the different ways. In addition, the two forms of non-associative learning appear to rely on different molecules in the nervous system. Knowledge on non-associative learning can help us better understand and explain how animals learn, and understanding basic forms of learning can help us unravel the mechanisms for more complex forms of learning.
Preface

https://doi.org/10.1093/oxfordhb/9780190456757.013.31. I was the first author of this book chapter, and conducted all of the literature research and wrote the original draft under the guidance of my supervisor Dr. Rankin. Section 1.4.3 is based a manuscript submitted to Current Protocols in Neuroscience as Yu, A.J., McDiarmid, T.A., Ardiel, E.A., and Rankin, C.H. (2018). High-throughput Analysis of Behavior Under the Control of Optogenetics in Caenorhabditis elegans. I was the co-first author of the manuscript, and wrote the original draft on the method part of it. For both parts, I have moderately reworded the writing and revised the content to fit into the scope of this thesis.

All experiments were conducted in Dr. Rankin’s lab at the Djavad Mowafaghian Centre for Brain Health at UBC. I designed and performed all experiments, and analyzed the data in experiments presented in Chapters 2 and 3. I independently worked on two of the genes reported in Chapter 3 (flp-20 and frpr-3), and brought the data to a multi-lab collaboration. The collaborative work was published as Chew, Y. L., Tanizawa, Y., Cho, Y., Zhao, B., Yu, A. J., Ardiel, E. L., … Schafer, W. R. (2018). An Afferent Neuropeptide System Transmits Mechanosensory Signals Triggering Sensitization and Arousal in C. elegans. Neuron, 0(0). 
https://doi.org/10.1016/j.neuron.2018.08.003. The published work of this collaboration focused on sensitization, the behavioural measures of sensitization reported in the publication were different from the behavioural measures I reported in this thesis.
Figure 10a was adapted from a figure in Thompson, R. F. (2009). Habituation: A history.

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List of Abbreviations

5-HT: serotonin
ANOVA: analysis of variance
ATR: All Trans-Retinal
cAMP: cyclic AMP
*C. elegans*: *Caenorhabditis elegans*
ChR2: Channelrhodopsin-2
*E. coli*: *Escherichia coli*
GPCR: G protein-coupled receptor
LED: light-emitting diode
LG: lateral giant
MWT: Multi-Worm Tracker
NGM: Nematode Growth Medium
PDF: pigment-dispersing factor
PKA: protein kinase A
PKC: protein kinase C
RNAi: RNA interference
SEM: standard error of the mean
S-R: stimulus-response
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I owe my parents especially my mother Helen a heartfelt thank-you. They have always been a source of unconditional support, both morally and financially. I could not have been who I am without you. I hope I make you proud.
Dedication

To my pet poodle Annie, an incredibly intelligent dog who was the first to make me wonder how animals learn.

Annie passed away on March 2, 2018.
Chapter 1: Introduction

1.1 The Facilitatory Processes in Non-Associative Learning

Animals interact with their environment and need to change their behaviour accordingly. The processes/mechanisms by which changes in behaviour are mediated are defined as learning. Changes in behaviour allow animals to interact with their environment more successfully and more efficiently, therefore, learning is adaptive and crucial for survival.

Theorists characterize animal learning into two categories: associative learning and non-associative learning. Non-associative learning is thought to be the simplest form of learning that does not require associations to be established – animals learn to modulate their responses when stimuli are presented in certain patterns. Non-associative learning can be further divided into two forms: habituation and sensitization.

In habituation, the response to repeatedly presented stimuli decreases. Animals are constantly bombarded with environmental stimuli, and recurring stimuli carry less new information, and habituation is thought to help organisms reduce unnecessary responses and free up attentional/neural resources for other important events.

Sensitization alters organism’s behaviour in the opposite direction of the effect of habituation. In sensitization, the magnitude and/or the likelihood of responding increases following the presentation of a novel and/or strong stimulus or a train of weaker stimuli. This facilitatory learning process is thought to help animals become more vigilant and alerted to achieve behavioural goals such as promoting escape.

In studying habituation, facilitation of a habituated response is termed dishabitation. In dishabitation, a novel strong stimulus induces facilitation of the response above the habituated level and can go back to or above the baseline levels. Dishabitation is used as one of parametric
characteristics of habituation to rule out fatigue or sensory adaptation being responsible for the behavioural decrement (Thompson & Spencer, 1966; Rankin et al., 2009), and relatively few studies have attempted to study dishabituation as an independent phenomenon.

Stimuli are often presented in certain orders as a result of the event (e.g., a predator approaching the prey first generates auditory stimuli, then visual stimuli), being able to acquire information from the previous stimulus can warn the organism to be better prepared for upcoming stimuli. Similarly, because the environment is ever-changing, one learned pattern (e.g., repeated stimuli that lead to habituation) may no longer be the same at a later time, and the organism needs to recover from the old learning if new information is received (e.g., a dishabituating stimulus), to ensure a proper response is elicited by the original stimulus. Both non-associative forms of facilitation, sensitization and dishabituation, are critical and essential to animal’s survival, as they help the organism adjust its behavioural strategy by increasing its response to novel or arousing stimuli.

1.1.1 The Dual-Process Theory

While both are classified as non-associative learning, sensitization is distinguished from habituation in several aspects. While the degree of habituation increases with more trials, sensitization can be produced with a single trial. Habituation appears to be relatively specific to the stimulus and occur in the stimulus response pathway, whereas the effects of sensitization involve increased arousal and are seen across all responses in an organism. Habituation is better induced with weak stimuli, in contrast, sensitization is better induced with strong stimuli (Marcus et al., 1988). There is also experimental evidence to show the two forms of non-associative learning can be produced simultaneously. In habituation studies (e.g. Koopowitz,
1975; Lockery et al., 1985), it is not uncommon to observe signs of sensitization in the first few trials following the initial presentation of the stimulus, before animals fully habituate.

Several theories were proposed to explain these behavioural observations in non-associative learning studies (e.g., Sokolov’s stimulus-model comparator theory (1963), Wagner’s gnostic unit theory (1979)), and the most influential and perhaps the most successful theory is the Dual-Process Theory by Groves and Thompson (1970). The Dual-Process Theory suggests that a stimulus simultaneously elicits both habituation and sensitization processes in the organism, and the animal’s observed response is the net outcome of two processes. The extent of sensitization is positively correlated with the intensity of the stimulus, and sensitization also habituates with repeated stimuli. Further, the Dual-Process Theory hypothesizes that the habituation process occurs in the specific stimulus-response (S-R) pathways, whereas sensitization’s effect is organism-wide and is regulated by a “state system”. It is worth noting that Thompson views this state system as a reciprocal neural network that encompasses a number of synaptic connections onto neural components in the S-R pathway in his recent opinion (Thompson, 2009).

The Dual-Process Theory recognizes dishabituation as a special form of sensitization, that is, sensitization of a habituated response. A novel stimulus activates a sensitizing process in the state system, that facilitates either a naïve response in one S-R pathway, or a habituated response in another S-R pathway, thus, the two behavioural phenomena are produced by the same facilitatory process. However, this view has been challenged by several studies.

1.1.2 The Cellular and Molecular Mechanisms of Sensitization

Sensitization is arguably the best understood learning and memory process today. A great amount of work contributing to our understanding of the mechanisms of sensitization has been done in two major model systems, *Aplysia* and leeches. The mechanism of short-term
sensitization in *Aplysia* was the first described molecular mechanism for learning (Castellucci et al., 1982).

Kandel and colleagues first studied the sensitization of the gill-withdrawal reflex in *Aplysia*. An electrical shock to the tail or the body wall significantly increased the magnitude of the elicited reflex, and the evoked postsynaptic potentials at the sensorimotor synapse in the abdominal ganglion increased in animals showing behavioural sensitization (Carew et al., 1971).

The effect of a single shock tapers off after several minutes, and is called short-term sensitization. A form of sensitization that persists longer can also be induced. Training *Aplysia* with five spaced shocks in one day or over four days produced long-term sensitization lasting for days to weeks (Pinsker et al., 1973; Frost et al., 1985). An increase in synaptic transmission was seen in animals after long-term sensitization training (Frost et al., 1985).

Several studies in behaving *Aplysia* (Carew et al., 1971; Walters et al., 1983) and isolated ganglia (Hawkins et al., 1981) indicated that short-term sensitization of defensive withdrawal reflexes is mediated by the heterosynaptic facilitation of sensorimotor synaptic transmission. Such facilitation altered the properties of presynaptic sensory neuron. Shocking the animal or electrically stimulating the interneurons in the isolated ganglion resulted in a decreased outward K+ current (Klein & Kandel, 1980), broadened action potentials (Walters et al., 1983), and increased neurotransmitter release (Castellucci & Kandel, 1976) in the sensory neurons, all of which lead to presynaptic facilitation, suggesting that presynaptic plasticity underlies the memory for short-term sensitization.

In search for the biomolecules producing sensitization, several lines of evidence implicated the neuromodulator serotonin. Levenson et al., (1999) found that the level of 5-HT increased in the hemolymph in tail-shocked *Aplysia*, and the increase was correlated with the
magnitude of the sensitizing stimuli; nerve stimulation also produced elevated 5-HT release (Marinesco & Carew, 2002). Injecting 5-HT into naïve animals sensitized their response (Philips et al., 2011). Similarly, perfusing the isolated abdominal ganglion with 5-HT produced facilitation of the sensorimotor synapse (Brunelli et al., 1976; Walters et al., 1983). In contrast, depleting endogenous 5-HT pharmacologically abolished tail shock-induced sensitization (Glanzman et al., 1989). Further confirming the role of 5-HT, application of 5-HT to sensory neurons was shown to reduce the outward K\(^+\) currents (Klein et al., 1982; Siegelbaum et al., 1982), and application of a 5-HT receptor antagonist blocked the spike broadening in the sensory neurons (Mercer et al., 1991). Anatomical studies also supported this view as serotonergic interneurons and sensory neurons were found to contact each other (Zhang et al., 1991).

Serotonin signalling primarily occurs through its G protein-coupled receptors. One of the main downstream effectors of 5-HT, cyclic AMP, was strongly implicated in sensitization. Intracellular injection of cAMP into the sensory neurons exerted effects similar to those of administration of 5-HT: decremented K\(^+\) currents (Klein et al., 1982; Siegelbaum et al., 1982) and enhanced evoked potentials (Brunelli et al., 1976) were observed. Lowering endogenous cAMP levels in the animal inhibited both short-term sensitization and presynaptic facilitation (Belardetti et al., 1983). The effect of cAMP is mediated by cAMP-dependent protein kinase (PKA), as injecting the catalytic subunit of PKA into sensory neuron was sufficient to produce the facilitation of neurotransmitter release (Castellucci et al., 1980), and injecting an inhibitor of PKA blocked the presynaptic facilitation (Castellucci et al., 1982).

These data led to the first proposed cellular mechanism for learning. Behavioural short-term sensitization is mediated by presynaptic facilitation following the activation of 5-HT signalling pathway. A sensitizing stimulus causes the heterosynaptic interneuron to release 5-HT
onto the sensory neuron. Binding of 5-HT to sensory neuron receptors activates a G protein alpha subunit, adenylyl cyclase, and PKA, in a series of biochemical reactions. The catalytic subunit of PKA then can phosphorylate specific proteins to alter the functional properties of a subset of K⁺ channels in the sensory neuron causing broadening of the action potential. The increased duration of depolarization causes a greater influx of Ca²⁺ into the sensory neuron leading to more neurotransmitter release from that sensory neuron. Thus, presynaptic facilitation is achieved, leading to the sensitized response.

However, this model could not fully explain some observations in later experiments. At least three other ion conductances were shown to be modulated by 5-HT (Baxter & Byrne, 1990; Braha et al., 1990; Walsh & Byrne, 1989). Besides the K⁺ conductances exclusively modulated by 5-HT (Hochner & Kandel, 1992), another second messenger pathway was identified to modulate a Ca²⁺ current and a voltage-dependent K⁺ current. Spike broadening was contributed to by the cAMP/PKA pathway as well as a G protein signalling pathway involving diacylglycerol and protein kinase C (PKC) (Sugita et al., 1994). Angers et al. (2002) showed the Aplysia homolog of synapsin (apSyn), a synaptic protein known to interact with vesicles, was phosphorylated by 5-HT-induced PKA and MAPK activity. Serotonin promoted dissociation of apSyn and synaptic vesicles to mobilize vesicles into the readily releasable pools.

A revised model of short-term sensitization was proposed based on the new findings (Angers et al., 2002). In addition to increasing the spike duration trough modulating K⁺ and Ca²⁺ conductances, 5-HT also increases mobilization of vesicles into the readily releasable pools by phosphorylating synapsin. Both processes are PKA-dependent, while PKC also plays a role. Through these mechanisms short-term sensitization is mediated by facilitated synaptic release in the sensory neuron.
The cellular and molecular mechanisms for long-term sensitization have also been thoroughly studied. Because this research focuses on short-term facilitation, I will summarize the current understanding of the mechanisms of long-term sensitization briefly.

There are both pre- and postsynaptic mechanisms for long-term sensitization. On the presynaptic side, similar to the mechanisms of short-term sensitization, altered the K⁺ channel conductance (Scholz & Byrne, 1987) and sensory neuron excitability (Cleary et al., 1998) were found. Increases in the number and size of active zones and vesicles, and more varicosities contacting the gill motor neurons were also observed in presynaptic sensory neurons were also observed in *Aplysia* trained for long-term sensitization (Bailey & Chen, 1983, 1988a&b, 1989)

Postsynaptic plasticity is also involved in the mechanism for long-term sensitization. Cleary et al. (1998) found the excitability of motor neurons increased in long-term sensitized animals. More excitatory receptors were found in the motor neuron after induction of long-term facilitation (Trudeau & Castellucci, 1995).

In long-term sensitization, structural plasticity in the presynaptic neuron requires the presence of the postsynaptic neuron (Glanzman et al., 1990). Experiments implicated an NCAM-related adhesion molecule in the presynaptic neuron (Mayford et al., 1992) and neurexin/neuroligin complex (Choi et al., 2011) in the neuronal outgrowth associated with long-term sensitization.

Long-term sensitization is also dependent on protein synthesis and changes in gene expression. Inhibiting protein and RNA synthesis were shown to block long-term facilitation of sensorimotor synapses and sensitization of withdrawal reflexes (Castellucci et al., 1986; Castellucci et al., 1989; Schacher et al., 1988). Protein synthesis in long-term facilitation is modulated by cAMP-responsive element-binding proteins (CREBs), a class of transcription
factors regulating gene expression (Kaang et al., 1993). Genes encoding (ApUch; Chain et al., 1999), transcription factors (Bartsch et al., 2000; Kim et al., 2003), growth factors (Kassabov et al., 2013) were all found to be differentially regulated in long-term sensitized animals.

Taken together, studies in Aplysia show that sensitization, depending on the time course of the memory, is mediated by heterosynaptic facilitation with different cellular mechanisms. Short-term sensitization is expressed in the presynaptic cells in a covalent modification manner, whereas long-term sensitization involves both pre- and postsynaptic changes, and often rely on more complex second messenger pathways including protein synthesis and gene expression.

Research investigating sensitization has also been carried out in medicinal leech Hirudo medicinalis. Boulis and Sahley (1988) studied sensitization of the shortening reflex in semi-intact leeches. A noxious stimulus did not increase the baseline shortening response; however, following the presentation of the noxious stimulus, normal habituation of the reflex to an innocuous stimulus was prevented, and a dishabituating stimulus after habituation training evoked an above-baseline response. Lockery and Kristan (1991) examined the leech local bending reflex. Leeches will bend several adjacent segments if a sensory cell for pressure (P cell) is repeatedly stimulated or a nociceptive cell (N cell) is stimulated in a local segment. Behaviour and electrophysiological recordings were done simultaneously. Sensitized animals bent their bodies with larger tension, and evoked motor neuron responses contained more spikes for local bending. Swim induction could also be sensitized by a noxious mechanical stimulus, as the latency for the leech to initiate swim shortened (Zaccardi et al., 2001). Interestingly, with repetition this sensitizing stimulus became less and less effective, showing habituation of sensitization.
Search for the locus of sensitization in leech has identified the S cell – the same interneuron implicated in habituation (Sahley et al., 1994). Increases in S cell activity were recorded in sensitized animals, and ablating S cells eliminated sensitization. Separating S cells from one another in the ventral nerve cord disrupted sensitization while the shortening reflex remained intact, suggesting the connections between S cells are important for sensitization (Modney et al., 1997).

The same neurotransmitter as in *Aplysia*, 5-HT, was shown to be critical for sensitization in leech. Intracellular stimulation of serotonergic neurons mimicked the sensitizing effect of shocks (Lockery & Kristan, 1991). Bathing the abdominal ganglion in 5-HT also increased the excitability of motor neuron (Burrell et al., 2001). Depletion of 5-HT disrupted sensitization (Ehrlich et al., 1992). In leech, 5-HT signalling cascade also recruits cAMP to induce sensitization (Zaccardi et al., 2004). These findings conform to the well-established *Aplysia* model.

One novel aspect of sensitization was demonstrated in leech. Burrell and Sahley (1998) observed a type of behavioural facilitation that showed proximity-dependent generalization: the extent of the facilitation is proportional to the distance between sensitized and tested sites. Later they found that this process was not mediated by 5-HT (Burrell & Sahley, 1999). These data pointed to a possible serotonin-independent form of sensitization.

Sensitization has also been studied in Crustacea. Krasne and Glanzman (1986) studied the sensitization of the lateral giant (LG) fibre-mediated escape response in crayfish. Following a strong electric shock to the body, the threshold for a test stimulus to elicit the LG response was significantly decreased. Crab escape responses could be sensitized by 5-HT in a dose-dependent
fashion: a low dose induced short-term sensitization and a high dose induced 24 h long-term sensitization (Aggio et al., 1996).

In summary, many species show sensitization in multiple behaviours for various durations. The enhancement of behavioural responses is mediated by synaptic facilitation. Memories for sensitization of different time courses are mediated by different signalling pathways, many of which use serotonin as the primary neurotransmitter.

1.1.3 Mechanism of Dishabituation Remains Elusive

As mentioned earlier, research focusing on dishabituation is rare, therefore, little is known about whether dishabituation and sensitization share the same cellular and molecular mechanisms. Having this piece of information would be crucial to determine whether the sensitization and dishabituation are the same or different facilitatory processes.

While the focus was still on sensitization, a few studies also looked into dishabituation and compared the two forms of facilitation; much of the data suggested that the two forms of facilitation are not identical. Rankin and Carew (1988) reported that sensitization and dishabituation emerge at different developmental stages in Aplysia larvae. Dishabituation can be produced at a younger stage, whereas sensitization can only be produced at an older stage. Also, when young Aplysia only shows dishabituation, the response facilitation increases only to the naïve baseline; however, in older Aplysia, dishabituation often produces a facilitation above the naïve baseline (Marcus et al., 1988). Sugita et al. (1997) found that facilitation of a depressed synapse (correlated with behavioural habituation) recruited a PKC-dependent process to a greater extent compared to synaptic facilitation at baseline (correlated with behavioural sensitization). The relative contribution of the PKA- and the PKC-dependent processes in the facilitation of a previously depressed synapse differs from that of a not depressed synapse, and this difference is
thought to make the differentiation between dishabituation and sensitization (Sugita et al., 1997). In leeches, ablating S cells only reduced dishabituation but not sensitization, suggesting that dishabituation and sensitization are two separate processes mediated by partly shared cellular pathways (Modney et al., 1997).

Gingrich and Byrne (1985) mathematically modelled a sensory neuron undergoing 5-HT-induced facilitation, and predicted the existence of a spike-duration independent process. The model was supported by experimental observations (e.g., Braha et al., 1990; Klein, 1993; Pieroni & Byrne, 1992), and it was hypothesized that an increased number of neurotransmitter vesicles moving from the storage pool to the readily-releasable pool was underlying facilitation from a habituated state. Hochner et al. (1986) suggested that action potential broadening by K+ channel closure in the presynaptic neurons seemed to be a specific mechanism for sensitization. In their experiment, the membrane potential of neurons was held at a level to prevent action potential broadening using voltage clamp. Under such condition, application of 5-HT was still capable of producing facilitation in a depressed synapse, but no longer effective in a normal synapse. This finding suggests that some additional processes were underlying dishabituation to produce the facilitation. The fact that there are several other 5-HT-modulated ion channel conductances (Hochner & Kandel, 1992) points to the possibility that sensitization and dishabituation may be mediated by two different facilitatory processes.

With limited evidence on the mechanism(s) of dishabituation, the current consensus generally is that they depend on molecular pathways that are partially shared with sensitization, but with distinct components (as reviewed in Byrne & Hawkins, 2015). More work is required to better characterize and differentiate the two facilitatory processes.
1.2 *C. elegans* as Model to Study Learning and Memory

The free-living nematode *Caenorhabditis elegans* was first chosen by Sydney Brenner (1974) as a model organism to study genetics and development. Fifty years of research has made *C. elegans* the best understood multicellular organism. The *C. elegans* genome was fully sequenced just two decades ago (*C. elegans* Sequencing Consortium, 1998), the cell lineage of the organism is invariant (Sulston et al., 1983), and the synaptic connections between its 302 identified neurons are largely conserved (White et al., 1986). This animal model has many clear advantages for simplifying the experimental work, while applying cutting-edge techniques.

Rankin, Beck and Chiba (1990) were the first to demonstrate that this “hard-wired” organism is capable of learning by reporting *C. elegans* could habituate, dishabituate, sensitize and show long-term memory for habituation. Since then, habituation have been well studied in *C. elegans* (as reviewed in Yu & Rankin, 2017). However, sensitization and dishabituation were not studied to the same extent as habituation in *C. elegans*, and relatively little work has focused on sensitization and dishabituation. Sensitization of the tap-withdrawal response was induced by a stronger stimulus in the form of a train of taps, and the magnitude of the reversal response was significantly increased compared to a naïve response (Rankin et al., 1990). Dishabituation was often tested in habituation assays to confirm the response decrement was a result of learning.

Mutations can cause deficits in both habituation and dishabituation. A mutation in vesicular glutamate transporter, EAT-4, was shown to affect both habituation and dishabituation of the tap-elicited reversal response (Rankin & Wicks, 2000). The findings suggested that modulation of neurotransmitter release could be involved in dishabituation. To systematically study and further our understanding of sensitization and dishabituation, it calls for a more detailed characterization of these facilitatory processes in *C. elegans*. 
Since that first report of learning, studies have found that this “simple” animal learns in many complex ways (as reviewed in Ardie & Rankin, 2010). Many sophisticated tools (e.g., transgenesis, Mello et al., 1991; RNA inference, Fire et al., 1998; optogenetics, Nagel et al., 2005) were developed and resources shared in the *C. elegans* research community have assisted and advanced investigations of the neurobiology of learning and memory in *C. elegans*.

*C. elegans* provides a versatile platform to conduct research to investigate the genetic and neural underpinnings of behaviour in a controlled, cell-specific, and reproducible fashion. With precise genetic manipulation, gene expression can be altered in specific cells or tissues to introduce or deplete proteins of interest. Also, because all neurons in the worm are identifiable, genetic and molecular mechanisms of behaviour can be studied at a single-cell resolution level.

Behavioural research in *C. elegans* also greatly benefits from its short life cycle, large brood size, and identical genetic background in every member of the animal. In our lab, we developed a high-throughput machine vision-based behavioural tracking system, the Multi-Worm Tracker (MWT; Swierczek et al., 2011). This system is capable of tracking up to 300 worms simultaneously in real time, and off-line analyses can generate detailed behavioural data with up to 26 raw individual parameters for parametric analyses. With the MWT, we can quantitatively and unbiasedly study learning and memory in a large population of *C. elegans*.

### 1.3 Objectives and Rationale

In this thesis, my goal was to address this central question: are sensitization and dishabituation mediated by the same or different facilitatory processes? I planned my research using behavioural and genetic approaches. I had two specific objectives designed to answer this question.
In the first objective, I characterized behavioural sensitization and dishabituation of an escape response facilitated by a mechanosensory stimulus in *C. elegans*, in order to find the behavioural paradigms that best produce these two forms of behavioural plasticity. I also used different behavioural paradigms to examine whether sensitization and dishabituation could be produced independently; if so, it would suggest that sensitization and dishabituation can be dissociated.

In the second objective, I used a candidate gene approach to investigate whether sensitization and dishabituation have the same or different genetic underpinnings. The rationale is that if mutations in genes differentially cause deficits in sensitization and dishabituation, it suggests that the two forms of behavioural changes are mediated by two separate facilitatory processes.

Understanding these facilitatory processes will help us better understand the interactions between different forms of non-associative learning processes that contribute to the animal’s overall behavioural state. Careful exploration and characterization of individual learning processes will help us establish a theoretical framework to integrate these forms of non-associative learning, in order to better explain and predict animal’s behaviour.

1.4 General Methods

1.4.1 *C. elegans* Strains

All *C. elegans* worms were maintained on Petri plates filled with Nematode Growth Medium agar (NGM), and *E. coli* (OP50) bacterial food was provided on the NGM plates as described in Brenner (1974).
The following two ChR2 transgenic strains were used as controls (referred to as “controls” from now on) in the work presented in this thesis:

VG61 \textit{lite-1}(ce314); \textit{yvIs1}[\textit{srp-6p::ChR2::YFP} + \textit{unc-122p::GFP}];

AQ2755 \textit{lite-1}(ce314); \textit{ijIs124}[\textit{gpa-13p::FLPase} +; \textit{sra-6p::FTF::ChR2::YFP}];

In VG61, ChR2 is strongly expressed in ASH, and weakly expressed in ASI and PVQ. In AQ2755, ChR2 is exclusively expressed in ASH.

The following strains were made by crossing canonical mutant alleles into the VG61 background, and homozygous transgenic mutant offspring were selected:

VG380 \textit{pdf-1}(tm1996); \textit{lite-1}(ce314); \textit{yvIs1};

VG382 \textit{pdf-2}(tm4393); \textit{lite-1}(ce314); \textit{yvIs1};

VG383 \textit{pdfr-1}(ok3425); \textit{lite-1}(ce314); \textit{yvIs1};

VG393 \textit{pdf-1}(tm1996); \textit{pdf-2}(tm4393); \textit{lite-1}(ce314); \textit{yvIs1};

VG524 \textit{gpc-1}(pk298); \textit{lite-1}(ce314); \textit{yvIs1};

The following strains were made by crossing canonical mutant alleles into the AQ2755 background, and homozygous transgenic mutant offspring were selected:

VG266 \textit{frpr-3}(gk240031); \textit{lite-1}(ce314), \textit{ijIs124};

AQ2786 \textit{flp-20}(ok2964); \textit{lite-1}(ce314), \textit{ijIs124};

AQ4133 \textit{frpr-3}(ok3302); \textit{lite-1}(ce314), \textit{ijIs124};

1.4.2 Behavioural Tracking on the Multi-Worm Tracker

All of my behavioural experiments were performed on the MWT. A standard optogenetic experiment protocol was used as described in Yu et al. (2018).

For optogenetic experiments, because \textit{C. elegans} does not synthesize all trans-retinal (ATR), the co-factor of ChR2, exogenous ATR must be provided. ATR was mixed into OP50 for
a 5 μM final concentration of ATR on the plate, and 50 mL of the food mixture was spread on the Petri plates to grow for 24-48 hours before use (Yu et al., 2018). For all experiments, worms were age-synchronized. To synchronize worms, five gravid adults were transferred onto the plates and allowed to freely lay eggs for 3-4 hours. After the egg-laying period, worms were picked off the plates, and plates were sealed with parafilm and stored until testing. This age-synchronization procedure typically produces 40-80 progeny worms on each plate. All worms were reared on the ATR plates, and kept in a dark drawer at 20 °C to minimize the light exposure to ATR, which causes isomerization of the ATR molecule. All worms were tested at 4 d old.

The MWT was used to deliver two types of stimuli with precise onset and offset that were programmable with the MWT software (v1.2.0.2; Swierczek et al., 2011). An electromagnetic solenoid (#195205-127, Ledex, Vandalia, OH, USA) was used to deliver tap stimuli at a distance of 0.583 mm away from one side of the Petri plate. A custom-built LED light apparatus (with six 1 w Luxeon LEDs) was used to deliver blue light stimuli to optogenetically activate ChR2 (Yu et al., 2018). The LED light apparatus was capable of delivering uniform blue light stimuli with a peak wavelength of 470 nm at an optical power density of ~70 μw/mm². An orange filter was installed on the camera lens to prevent blue light from entering the camera.

1.4.3 MWT Data Analysis

Data collected by the MWT were first analyzed using Choregraphy (v1.3.0_r1035; Swierczek et al., 2011) to generate data on reversal response. In this step, “—shadowless”, “—minimum-move-body 2”, and “—minimum-time 20” filters were applied to restrict the analysis to animals that moved at least two body lengths and were continuously tracked for at least 20 s, and the MeasureReversal plugin was used to quantify reversals with a criterion of “dt=3”, which
recognized all backward movement within 3 s of the light pulse onset as reversal responses elicited by the stimuli; a criterion of “dt=1” was used to recognize backward movement within 1 s of stimulus onset as reversal response elicited by the tap. Several custom Matlab (MathWorks, 2015b) scripts were used to organize and summarize Chorepgraphy output for graphing and statistical analysis.

Statistical analysis for all experiments was performed using Matlab (MathWorks, 2015b). In general, comparisons between two groups were performed using paired or unpaired t-tests depending of the nature of the data. Comparisons between multiple groups tested with a single strain or condition were performed using a one-way ANOVA with Tukey’s multiple comparisons post hoc test. Comparisons between multiple groups tested with multiple strains or conditions were performed with a two-way ANOVA, and if a significant main effect was found, follow-up one-way ANOVAs with Tukey’s multiple comparisons post hoc test were performed. For all statistical analyses, alpha was 0.05.
Chapter 2: Behaviourally Characterizing and Dissociating Sensitization and Dishabituation in *C. elegans*

2.1 Introduction

The Dual-Process Theory considered both sensitization and dishabituation as the same facilitatory process. The only difference was that the two behavioural phenomena are exhibited in different behavioural state backgrounds (naïve vs. habituated), and the effect of this facilitatory process on the animal’s response is observed as sensitization, if the response is elevated above the naïve response level, or dishabituation, if the response recovers from the habituated response level, but does not exceed the naïve response level. However, an alternative possibility is that dishabituation is a second facilitatory process independent of sensitization that specifically reverses habituation. One way to answer this question is to determine whether it is possible to dissociate sensitization and dishabituation.

Some studies do show that sensitization and dishabituation can be differentiated in several aspects. As previously mentioned, dishabituation develops at an earlier life stage and sensitization develops later in *Aplysia* (Rankin & Carew, 1988). Marcus et al. (1988) showed that when sensitization is fully developed, a dishabituating stimulus often produces facilitation above the naïve baseline (Marcus et al., 1988). This above-baseline facilitation produced by a dishabituating stimulus has also been observed in several other species (e.g., medicinal leech, Alkatout et al., 2007; crayfish, Krasne, 1978; spinal rat, Thomspnon & Spencer, 1966). Marcus et al. (1988) also found that sensitization and dishabituation were best produced by different stimulus intensities. In addition, a more recent study using reduced preparation of *Aplysia* suggested that dishabituation may involve either reversal of habituation or sensitization.
depending on the sensory modality and time course used in the experimental paradigms (Hawkins et al., 2006).

These lines of evidence suggest that: 1) sensitization and dishabituation are potentially two separate facilitatory processes relying on developmentally different mechanisms, and 2) sensitization and dishabituation may occur simultaneously and/or independently in different paradigms. I therefore focused my research on dissociating the two processes, and I began by characterizing the two behavioural phenomena in *C. elegans*, in order to establish behavioural paradigms to study the facilitatory processes underlying the behavioural changes.

In most studies of sensitization and dishabituation the facilitation of the response to stimuli in one sensory modality is produced by stimuli in another sensory modality. In our lab, we have established a method to stimulate two sensory modalities in a population of *C. elegans*. I used this method and developed paradigms to produce sensitization and dishabituation in worms. *C. elegans* naturally responds to mechanosensory taps, and it can also respond to blue light if a genetically encoded light-gated cation channel, Channelrhodopsin-2 (ChR2), is expressed in the sensory neurons. Using the Multi-Worm Tracker, tap and light stimuli can be delivered discretely and simultaneously to virtually all animals on a Petri plate to elicit behavioural responses in worms. The MWT can control tap and light stimulus delivery with precise onset and offset, thus, minimizing the interference from one stimulus to the next. In this study, I used worms with ChR2 expressed in the ASH pair of nociceptive neurons in the head region of the worm. We previously showed that the ASH ChR2-mediated reversal response could habituate and dishabituate after a tap stimulus (Ardiel et al., 2016, 2017), thus, this response is the focus of this series of experiments that examine tap-induced sensitization and dishabituation of the optogenetic ASH reversal response.
Based on previous research and results from pilot studies, my hypotheses for this series of experiments were: 1) a tap stimulus will induce facilitation of light-evoked ASH responses, in both naïve and habituated animals, observed as sensitization and dishabituation of the ASH response, and 2) the tap-induced facilitation of ASH response will show a time-dependent effect.

2.2 Methods

2.2.1 C. elegans Strain

AQ2755 was the only strain used to perform the experiments in this chapter. This transgenic strain expresses ChR2 only in ASH.

2.2.2 Behavioural Paradigms

Two separate behavioural paradigms were designed to produce sensitization and dishabituation. Depending on the objective for an experiment, either mechanosensory taps or 2 s blue light pulses were used in a sequence that would lead one to produce facilitation in the response to the other.

In the sensitization paradigm, a plate of worms was placed on the tracker for 300 s to allow them to return to their behavioural baseline after being moved onto the tracker platform (Giles, PhD dissertation). After 300s, a sensitizing stimulus (tap or light pulse) was delivered to sensitize the animals. After an interval ranging from 10 to 300 s following the sensitizing stimulus, a test stimulus was administered to elicit a reversal response which was later quantified. For each time interval tested for sensitization (10, 20, 30, 40, 60, 120, and 300 s), a control group was tested in which the sensitizing stimulus was omitted and the test stimulus was delivered at a matched time point to acquire the naïve response level.
In the dishabituation paradigm, animals were given a 300 s acclimatization period on the tracker before habituation training began. Thirty stimuli (tap or light pulse) were delivered every 10 s to complete habituation training, then a dishabituating stimulus (tap or light pulse) was administered 10-300 s following the last stimulus in habituation training to dishabituate the response. Responses to the habituated stimulus after the dishabituation stimulus were compared to the responses in the spontaneous recovery control group, and responses for both groups were compared to the final level of habituation.

2.2.3 Data Analysis

For sensitization experiments with multiple intervals, the four response components were compared using one-way ANOVAs with Tukey’s multiple comparisons post hoc test between different groups.

For dishabituation experiments with multiple intervals, in all four response components, habituation in each group was first confirmed by comparing the initial and final response levels using a paired t test. Then, the response components were compared using one-way ANOVAs with Tukey’s multiple comparisons post hoc test between different groups.

For sensitization and dishabituation experiments with only one interval, response components were compared using an unpaired t test with a Bonferroni correction for multiple comparisons. For all statistical analyses, alpha was .05.
2.3 Results

2.3.1 Characterizing Tap-Induced Sensitization and Dishabituation of ASH-Mediated Reversal

In Ardie et al. (2016), we reported that the habituated response of ASH-mediated reversal could be dishabituated by a mechanosensory tap administered 10 seconds after the last stimulus in habituation training. To examine and capture the dynamic changes of the tap’s effects on sensitizing the naïve ASH reversal response, and dishabituating the habituated ASH reversal response, I designed a series of sensitization and dishabituation learning paradigms to characterize the behavioural changes over time.

2.3.1.1 Characterizing Tap-Induced Sensitization

In this experiment, I manipulated the interval between the sensitizing stimulus (a tap) and the test stimulus (a 2 s blue light pulse activating ChR2 in ASH neurons), and examined in which response components behavioural changes were produced, and how long the sensitizing effect lasted. I tested seven post-tap intervals (10, 20, 30, 40, 60, 120, and 300 s), and control groups run at the same time did not receive the tap, but did receive the blue light at the same time interval. making a total of 14 groups, with each group consisting of six plates of 40-80 worms.

The results illustrated the dynamic changes in the sensitization of the ASH response produced by a tap 10–300 s prior to optogenetic activation of ASH (Figure 1). I measured four different response metrics of the ASH-mediated reversal: response probability, response speed, response duration, and response latency. For each plate, response probability was calculated as the number of worms that changed their direction of movement to backward within 3 s of the light stimulus onset. If a worm reversed to the stimulus, its response speed, duration, and latency were quantified. Average response speed, duration, and latency for each plate were calculated
using the data of all worms that responded to the blue light stimulus. Plate data were grouped
together, and the sample size was the number of plates in each group. As shown in Figure 1, a
tap produced facilitation in all four response components when the sensitized responses were
significantly larger than the naïve response levels at the same interval, but interestingly, different
sensitization effects in these four metrics were observed.

For response probability, a main effect was found, $F(13, 77)=9.45, p<.001$, and multiple
comparisons test revealed that sensitization was significant at 10, 20, 30, 40, 60, and 300 s (all
$p$s $<.05$). The facilitatory effect was not statistically significant at a 120 s interval ($p=.35$).

For response speed, a significant main effect was found ($F(13, 77)=10.07, p<.001$).
Multiple comparisons showed the facilitatory effect was significant at 10, 20, and 30 s intervals
(all $p$s $<.05$), but was no longer significant after 40 s (for the comparisons between naïve and
sensitized response speeds at 40, 60, 120, and 300 s, $p$s $=.65, .10, .58, .42$).

For response duration, an ANOVA showed a significant main effect ($F(13, 77)=7.95,
$p<.001$), and the post hoc test found significant tap-induced sensitization at 10, 20, 30, and 40 s,
intervals (all $p$s $<.05$). The sensitization was not significant at 60, 120 or 300 s ($p$s $=.16, .92, .98$).

Response latency was plotted in its multiplicative inverse such that a higher bar
represents a shorter latency; a significant main effect was found, $F(13, 77)=2.28, p=.014$. The
post hoc test showed that shortened response latency produced by the tap (presented as a higher
bar in the graph) was only observed at the 10 s interval ($p<.05$). At other intervals (20, 30, 40,
60, 120, and 300 s), there was not a significant effect of the tap-induced sensitization.
Figure 1 Characterization of the tap-induced sensitization of ASH response in four response metrics. The behavioural paradigm used in characterizing the effect of tap-induced sensitization is provided. Four response metrics of the ASH-mediated reversal, response probability, speed, duration, and latency, showed facilitation above the baseline with different profiles. Black bars are the naïve response levels, and red bars are the tap-sensitized response levels; a red bar significantly higher than its paired black bar indicates that significant sensitization was observed at the given time point. The effects of tap-induced sensitization on probability and duration lasted at least 60 s, whereas speed and latency had shorter periods of sensitization. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
2.3.1.2 Characterizing Tap-Induced Dishabituation

In this experiment I investigated the time course of tap-induced dishabituation. I used the same time intervals (10, 20, 30, 40, 60, 120, and 300 s) as in the previous experiment to quantify the dynamic effect of tap-induced dishabituation of blue light ASH habituation. The results showed that the four parameters of the ASH response I examined showed different patterns of facilitation (Figure 2).

Groups were considered habituated if the final response level was significantly lower than the initial response level, dishabituation was considered to have occurred if the response after tap was significantly above the habituated level and sensitization was considered to have occurred if the response after tap was significantly above the baseline response level. In all groups, duration habituated (paired t test, \( df=5, ps<.05 \)), but the three other components did not always significantly decrease. Four groups for response probability, four groups for response speed, and five groups for response latency out of the 14 groups did not decrement to levels that were significantly lower than initial response levels. Data on habituation training from all groups were combined and the population initial and final response levels of the four response components were plotted in dashed lines for visualization. As shown in Figure 2, the ASH response was facilitated by the tap in some response components, however, the details and dynamics were not the same across the components.

For habituation of response probability, a significant effect was found between different groups, \( F(13,77)=7.21, p<.001 \). Multiple comparisons found that the tap stimulus produced significant facilitation at the 10, 20, 30, 40, and 120 s intervals (all \( ps<.001 \)). No significant differences were found at the 60 and 300 s intervals (\( ps=.077, .116 \)).
For response speed, a significant effect was found by ANOVA ($F(13,77)=2.3, p=.013$). The only significantly different spontaneous recovery and dishabituation groups were at the 300 s interval ($p=.010$). A trend at the 10 s interval was observed with a $p=.056$. No significant differences between spontaneous recovery and dishabituation groups were observed other intervals (all $ps>.05$).

For response duration, a significant effect was found, $F(13,77)=2.39, p=.010$. Examining the data with multiple comparisons showed that a significant facilitation was seen at the 10, 30, and 60 s intervals ($ps=.001, .031, .029$). Response facilitation at other intervals was not statistically significant (all $ps>.05$).

Latency was plotted in its multiplicative inverse, therefore, a higher bar in the figure indicates a shorter latency; habituation of the ASH response latency results in an increase in this response component (Ardiel et al., 2016). If response latency increases, a shorter bar should be observed, and if response latency is dishabituated by the tap, a significantly higher blue bar than the black bar should be observed. For response latency, a significant effect was found by an ANOVA ($F(13,77)=2.17, p=.020$), however, further analysis with multiple comparisons revealed that the significant difference between spontaneous recovery and dishabituated groups at the 10 s interval was opposite to the pattern of dishabituation (the blue bar was shorter than the black bar). There were no significantly different groups at other intervals (all $ps>.05$).
Figure 2 Characterization of the tap-induced dishabituation of ASH response in four response metrics. The behavioural paradigm used in characterizing the effect of tap-induced dishabituation is provided. Four response metrics of the ASH-mediated reversal, response probability, speed, duration, and latency, showed facilitation in forms of sensitization and dishabituation differentially. Black bars are the spontaneous recovery response levels, and blue bars are the tap-dishabituated response levels; a blue bar significantly higher than its paired black bar indicates that significant dishabituation was observed at the given time point. The effects of tap-induced sensitization on probability and speed lasted at least 40 s, whereas duration showed dishabituation that lasted at least 30 s. No facilitation was statistically detected in response latency. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
2.3.1.3 Summary of Characterizing Tap-Induced Response Facilitation

As seen in Figures 1 and 2, ASH response facilitation can be produced by a tap stimulus in all four reversal response components. Different response components showed different dynamic changes to the tap. The dishabituation paradigm produced above-baseline, sensitization-like facilitation in response probability and speed, while response latency was not sensitive to tap-induced facilitation. Of all four components of the ASH response, response duration showed the most robust and consistent effect: significant sensitization of naïve ASH response duration, habituation of the ASH response duration, and dishabituation of habituated ASH response duration were all clearly observed. Based on these results, I decided for future genetic experiments I would focus on sensitization and dishabituation of response duration tested at a 10 s interval.

2.3.2 Exploring the Effect of Behavioural Paradigm on Facilitation

In the previous two experiments, the behavioural effects of the tap stimulus on naïve and habituated ASH response durations showed sensitization and dishabituation. In Figures 2 and 3, tap-induced sensitization and dishabituation of ASH response duration showed similar dynamic changes: in the beginning, a large facilitation was observed, then the facilitatory effect gradually waned, and was not significantly different from spontaneous recovery after 60 s. The similarity in the pattern of these two facilitatory processes again leads to the question as to whether they reflect the same underlying facilitatory process superimposed on different response levels (baseline or habituated) or whether there are other differences between the processes.

To investigate whether sensitization and dishabituation could be differentiated, I designed the following experiment. For sensitization I replicated sensitization paradigm I used in the previous experiment in which I delivered the sensitizing stimulus 10s after the 300s rest period.
For dishabituation I replicated the dishabituation paradigm I used in the previous experiment in which I delivered the dishabituating stimulus 10s after the final stimulus in habituation training, and then dishabituation was measured after an interval (I termed this post-training dishabituation). In this experiment, I designed a second dishabituation paradigm, in which animals were first given the designated post-habituation interval, and the dishabituating stimulus was delivered 10 s prior to the test stimulus (I termed this pre-testing dishabituation). In both dishabituation paradigms, spontaneous recovery of the habituated response would occur during the interval, but in the post-training dishabituation paradigm, the tap-induced facilitation might gradually taper off, whereas in the pre-testing dishabituation paradigm, the tap-induced facilitation should always be of the same magnitude despite the length of the interval. The patterns of facilitation produced by these two dishabituation paradigms was compared to the pattern of naïve response sensitization after a 10 s interval, to investigate whether the different forms of facilitation can be produced in the same habituated background by different behavioural paradigms.

For these sensitization and dishabituation paradigms, I chose three intervals, 10, 60, and 300 s. These three intervals were chosen because in my previous sensitization and dishabituation experiments, for response duration a large facilitatory effect observed at the 10 s interval, had started to diminish after the 60 s interval, and was completely gone after the 300 s interval.

A one-way ANOVA showed a significant effect ($F(9, 59)=15.7$, $p<.001$), and pairwise multiple comparisons between different groups confirmed the significance of a number of observations.

For sensitization, I first compared the naïve response duration and the response duration sensitized by a tap delivered 10 s earlier (Figure 3, open black and red bars). The one-way
ANOVA \((F(9, 59)=15.7, p<.001)\) and multiple comparisons between the naïve and sensitized responses found a significant difference \((p<.001)\).

Next, I compared the response durations in the spontaneous recovery and dishabituated groups at the 10, 60, and 300 s intervals.

For spontaneous recovery, with a one-way ANOVA \((F(9, 59)=15.7, p<.001)\) and multiple comparisons, it was found that at a 10 s interval after habituation training, the spontaneous recovery response duration was significantly lower than the naïve response duration \((p=.031)\); little spontaneous recovery of habituation would have occurred within a short time, therefore, duration of the ASH response habituated. At the 60 and 300 s intervals, spontaneous recovery response durations were no longer significantly different from naïve response duration \((ps=.876, .996)\), suggesting that the habituated response duration had recovered to a close-to-naïve response level after 60 s (Figure 3, solid black bars).

In the post-training dishabituation groups, a tap stimulus was always delivered 10 s after habituation training, and the facilitatory effect of the tap on the ASH response duration was examined at three different time points (Figure 3, solid blue bars). A one-way ANOVA showed a significant effect \(F(9, 59)=15.7, p<.001\). Multiple comparisons revealed that at a 10 s interval, the tap-dishabituated response duration was significantly longer than the spontaneous recovery response duration \((p<.001)\), showing tap-induced dishabituation. The tap-dishabituated response duration at a 10 s interval was not significantly different from the naïve response duration \((p=.876)\), indicating that the tap-induced dishabituation at a 10 s interval increased the habituated response duration back to the naïve response level. At the 60 and 300 s intervals, a post-training tap’s facilitatory effects on response duration were no longer statistically distinguishable from the response facilitation of spontaneous recovery \((ps=.888, .997)\), and the durations of the tap-
dishabituated response were not significantly different from the naïve response duration ($ps=1.000, 1.000$). Interestingly, the durations of the tap-facilitated ASH response measured at three post-training intervals were not statistically different from each other (for all pairwise comparisons, $ps>.05$), suggesting that the tap-facilitated response duration maintained at the same level or decreased very little over time. Meanwhile, spontaneous recovery progressed, and after a 60 s interval it had recovered response duration to a level that was not statistically different from the naïve response level. These results were generally congruent with the previously reported data shown in Figure 2.2.

In the new pre-testing dishabituation paradigm, animals were given three different spontaneous recovery intervals right after habituation training, then, a dishabituating tap stimulus was delivered after the intervals, followed by a test stimulus optogenetically activating ASH 10 s later (Figure 3, solid red bars). A significant effect was found by a one-way ANOVA ($F(9, 59)=15.7, p<.001$), and multiple comparisons were performed. The pre-testing and post-training paradigms at the 10 s interval were the equivalent (the tap stimulus was delivered 10 s after habituation training and 10 s before the test stimulus), therefore, as previously shown at a 10 s interval, the tap stimulus dishabituated the habituated response duration to the naïve response level. At a 60 s interval, a pre-testing dishabituating tap stimulus caused a sensitization-like facilitation in the ASH response duration; the dishabituated response level was significantly higher than the spontaneous recovery response level at the same time point ($p<.001$) and the naïve response level ($p<.020$). At a 300 s interval, tap-induced facilitation of the response duration was significant: the dishabituated response duration by a pre-testing tap stimulus was significantly longer than the spontaneously recovered response duration ($p<.001$). Similar to the pattern at a 60 s interval, this tap-facilitated response level was significantly higher than the
naïve response level \((p<.001)\). Interestingly, at the 60 and 300 s intervals, the response durations facilitated by a pre-testing tap stimulus were above the naïve baseline, and were not significantly different from the tap-sensitized response duration in the naïve background (for all pairwise comparisons, \(ps>.05\)).

Comparing the tap-dishabituated response durations in the post-training and pre-testing dishabituation paradigms, at a 10 s interval, because the two paradigms were identical, so were the data; at a 60 s interval, the pre-testing tap stimulus resulted in a significantly longer response duration than the post-training tap stimulus \((p=.018)\); at a 300 s interval, the response duration dishabituated by a pre-testing tap stimulus was significantly longer than the response duration dishabituated by a post-training tap stimulus \((p<.001)\).

Taken together these data, I found that the time point at which the dishabituating tap stimulus was delivered was critical in terms of the patterns of response facilitation that would be observed. Specifically, in naïve background and in backgrounds in which animals first habituated then spontaneously recovered for either a 60 s or a 300 s intervals, a tap stimulus delivered 10 s prior to a test optogenetic stimulus activating ASH facilitated the ASH response durations above the baseline to a similar level; tapping the animals right after habituation training led to a back-to-baseline dishabituation of the ASH response duration when tested at a 10 s interval, and the facilitatory effect probably waned, as spontaneous recovery also occur over time to facilitate the habituated response, and net behavioural effect of the two processes were observed at a level close to the naïve baseline when tested at the 60 and 300 s intervals.
Figure 3 Characterization of the tap-induced facilitation of ASH response in four response metrics with different paradigms. The behavioural paradigms used in comparing the facilitatory effects of different paradigms.

Three different paradigms, sensitization of a naïve response, dishabituation of a response with the dishabituating stimulus delivered either 10 s after training or before testing, were illustrated. For sensitization, open black bar is the naive tap response duration, and open red bar is the sensitized tap response duration; for dishabituation, solid black bars are the spontaneously recovered response durations at different time points, solids blue bars are response durations with a post-training dishabituation paradigm, and solids red bars are response durations with a pre-testing dishabituation paradigm. The effects of tap-induced sensitization and dishabituation on response duration were compared. A tap delivered 10 s before the test stimulus produced sensitization-like facilitation in response duration, whereas a tap delivered 10 s after habituation training produced dishabituation-like facilitation. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
2.3.3 Determining the Directionality of Facilitation

In the experiments previously described thus far, a tap facilitated both naïve and habituated ASH reversal responses. The next question I asked was that whether the converse was true, that activating ASH could sensitize and/or dishabituate naïve and habituated tap responses. To address this question, I modified the behavioural paradigms using blue light as the sensitizing/dishabituating stimulus and tap as the test stimulus, to measure the tap-elicited reversal responses in the presence and absence of the blue light stimulus in naïve and habituated animals.

I first tested whether a blue light stimulus to activate ASH could sensitize the reversal response to tap. After a 300 s acclimatization period, a 2 s blue light stimulus was delivered, then after a 20 s interval, a tap stimulus was delivered. A control group was run with only a tap at 320 s and without the blue light stimulus. Three response metrics of the tap-elicited reversal in the sensitized and naïve groups were compared. I observed that in none of the metrics were the tap response facilitated by optogenetic activation of ASH; for response probability $t(12)=1.097$, $p=.30$, for duration $t(12)=1.097$, $p=.30$, and for speed, $t(12)=.185$, $p=.86$, (alpha = .05/3 with a Bonferroni correction for multiple comparisons) (Figure 4a).

Next, I tested whether a blue light stimulus to ASH could dishabituate the reversal response to tap. Animals were first acclimatized to the tracker for 300 s, then a tap habituation training session began. Worms were trained with 30 taps at a 10 s inter-stimulus interval. 10 s after the last training stimulus, a 2 s blue light stimulus was delivered, followed by a tap test stimulus 10 s later. A group of control animals were trained for habituation in the same way, and the test stimulus was delivered at the same time point, but no blue light stimuli were delivered in between habituation training and the test stimulus. Both the control and the dishabituated groups...
habituated in three response components (paired t test, $df=5, ps<.05$). The response components were compared between the two groups with the naïve and habituated response levels plotted in dashed lines to visualize habituation and facilitation from habituated baseline (Figure 4b). I found that, like with the sensitization paradigm, an ASH stimulus was not able to produce dishabituation in any components of the tap response (for response probability $t(12)=1.152, p=.28$, duration $t(12)=.177, p=.86$, and for speed, $t(12)=.299, p=.77$ (alpha = .05/3 with a Bonferroni correction for multiple comparisons)).
Figure 4 ASH stimulation produces no sensitization nor dishabituation in tap-elicited reversal response. The behavioural paradigm used in characterizing the effect of tap-induced dishabituation is provided. Three response metrics of the tap reversal, response probability, speed, and duration, were examined. (a) Black bars are the naïve tap reversal response levels, and red bars are the optogenetic ASH-sensitized response levels; a red bar significantly higher than its paired black bar indicates that significant sensitization was observed at the given time point. Optogenetic activation of ASH led to no sensitization in tap response compared to naïve controls. (b) Black bars are the habituated tap response levels, and blue bars are the optogenetic ASH-dishabituated response levels; a red bar significantly higher than its paired black bar indicates that significant dishabituation was observed at the given time point. Mean +/- SEM of the population initial and final response levels during habituation were represented in dashed lines. Optogenetic activation of ASH led to no dishabituation in tap response compared to spontaneous recovery controls. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
2.4 Discussion

In this chapter, I performed several experiments to confirm my hypotheses. I found that a tap stimulus can induce sensitization and dishabituation of a ChR2-mediated ASH reversal response. The form of facilitation produced by a tap stimulus is dependent on the animal’s behavioural state (naïve or habituated), time, and the experimental paradigms used. I also found that a tap stimulus can produce facilitation in ASH response, but activation of ASH does not produce facilitation in tap response.

In characterizing the facilitatory effect of a tap stimulus on ASH response, I devised two paradigms intended to produce sensitization and dishabituation separately. My results showed that the facilitatory process(es) activated by tap had different effects on different response components. As shown in Figures 1 and 2, behavioural sensitization could be produced in four different response components of the ASH-mediated reversal, but behavioural dishabituation was only seen in response duration – the dishabituation paradigm produced above-baseline dishabituation, or sensitization, in response probability and speed, and had no effects on response latency. It is worth noting that habituation of response probability and speed were less pronounced than habituation of response duration, and the decrements from initial to final levels in these two response components were relatively small, perhaps the magnitude of the tap-induced facilitation was greater than the magnitude of the decrement (as a result of habituation), thus, the net behavioural effect was observed as a facilitation above the baseline. This suggests that sensitization and dishabituation may be simultaneously produced by a single tap stimulus, and the behavioural effect of the facilitatory processes can be observed as sensitization or dishabituation depending on the animal’s existing behavioural states. These results are also in line with previous studies in which above-baseline facilitation was produced by a dishabituating
stimulus. Response latency was the least sensitive response component to the facilitatory effect of the tap stimulus. Sensitization of a naïve ASH response by the tap was only observed at the shortest interval (10 s), and dishabituation of a habituated ASH response by the tap was not observed at any intervals. A possible explanation is that this was due to the ceiling effect in this response component. We previously demonstrated that a 2 s optogenetic stimulus mimics a natural osmotic pressure stimulus that triggers a response with a long latency (Yu et al., 2018).

ASH neurons are nociceptor neurons in worms to detect aversive and/or lethal stimuli, and an ASH response that occurs reliably and invariantly would be advantageous. Because of that, sensitization, habituation, and dishabituation induced with my experimental paradigms might not be obvious. A future study might use a weaker stimulus to produce a lower probability ASH response to test this hypothesis.

Because clear, robust, and consistent sensitization, habituation, and dishabituation of response duration could be produced by the two paradigms, future experiments would specifically examine this response parameter to determine whether manipulations could affect the facilitation produced in one paradigm but not the other, adding one piece of evidence to dissociate sensitization and dishabituation.

Different response components also showed facilitatory effects of different durations, and the forms of time-dependency of the same response components in sensitization and dishabituation paradigms did not fully agree with each other. These results suggested that there are multiple underlying facilitatory mechanisms for different components of the same response. In addition, I found that habituation of different response components did not always occur to the same extent: while response duration always significantly decreases in habituation, response probability, speed, and latency habituated less. Previously in our lab, it was also found that
different genes affect different tap response components in habituation (Giles, PhD dissertation). My findings together with Giles’ findings illustrated that the simple forms of learning are much more complex and sophisticated that can involve modulation and integration of many different genetic mechanisms.

Data in Figure 3 showed that different paradigms could result in different forms of facilitation being observed. The facilitatory effect on animal’s behavioural response is time-dependent: the facilitation was pronounced shortly after the tap stimulus (in the sensitization and pre-testing dishabituation paradigms) and gradually decreased in seconds to minutes (in the post-training dishabituation paradigm). In the post-training dishabituation paradigm, the dishabituating tap stimulus was administered immediately after habituation training, and if the facilitatory effect of the post-training tap did not dissipate over time, at the 60 and 300s intervals when spontaneous recovery occurred to greater extents, the net behavioural effects of a tap-induced facilitatory process and spontaneous recovery should show an additive effect and cause the ASH response duration to be above the baseline. If this was the case, it would suggest that dishabituation is a facilitatory process that specifically reverses habituation but does not produce sensitization. However, the fact that a tap produced facilitation in response duration of the same magnitude did not allow me to definitively differentiate sensitization and dishabituation – the same facilitatory process could be underlying the behavioural increment in either naïve or habituated response. Conducting behavioural experiments alone may not be fruitful in differentiating the two facilitatory processes, and other approaches must also be incorporated into the research.

The behavioural paradigms in this experiment only sampled the trajectory of the facilitatory effect at three different intervals. Maybe sensitization and dishabituation can be
differentiated at other time points that were not examined in this experiment. In future experiment, I can investigate this possibility by expanding both post-training and pre-testing dishabituation paradigms with more intervals, in order to capture the dynamic effects of the tap-induced facilitatory process(es).

The results in Figure 4 demonstrated that, although a tap stimulus can sensitize and dishabituate the ASH response, a stimulus to ASH neither sensitized nor dishabituated the tap response. The facilitatory capacity between the two sensory modality appears to be unidirectional. This suggests that the molecular mechanisms mediating these facilitatory processes are activated in a specific order – from touch receptor neurons to ASH neurons. This piece of information was helpful and guided me to formulate my next hypothesis in the experiments described in Chapter 3.

One caveat of this experimental design is that sensitization and dishabituation of tap response with ASH stimulation were only tested with one interval in each paradigm. It is possible that the ASH-induced facilitation has different time-dependent dynamics that are different from those of tap-induced facilitation. Future research should replicate the experiment with a range of between-stimuli intervals to more carefully examine whether ASH stimulation can sensitize or dishabituation tap-elicited reversal response.
Chapter 3: Differentiating the Genetic Components of Facilitatory Processes

3.1 Introduction

Behavioural changes are mediated by a series of extra- and intracellular events in the organism, that modulate the activity in the nervous system. Neuromodulatory molecules are heavily implicated in altering neuronal excitability and behavioural plasticity. These neuromodulatory molecules include monoamines, acetylcholine, and neuropeptides, that act through G protein-coupled receptors (GPCRs), to trigger intracellular signalling cascades that modulate the cell’s physiology. Because this metabotropic modulation does not directly gate ion channels, rather, it relies on a number of signalling pathways, the effect is not immediate and can be longer-lasting than the effects of classical neurotransmitters.

The neuromodulatory role of monoamines has been extensively studied in a number of species. The best understood mechanism of non-associative learning is the serotonin-mediated synaptic facilitation underlying behavioural sensitization in Aplysia. Short-term sensitization is achieved by the 5-HT signalling cascade in the sensory neuron that broadens the action potential spike and increases synaptic release onto the motor neuron (Castellucci & Kandel, 1976). Other studies have also unravelled the role of monoamines in simple forms of learning. For example, in C. elegans dopamine signalling has been shown to modulate the sensitivity of the anterior mechanosensory neurons, and affect the rate and level of tap habituation (Kindt et al., 2007). Octopamine is also shown to produce facilitation in a habituated response in locust (Sombati & Hoyle, 1984); interestingly, this facilitatory effect of exogenous octopamine was no longer observed after one minute.

Recently neuropeptides have become a hot research topic in C. elegans, and because of the large variety of the molecules and their signalling partners, their role in behavioural plasticity
is not well characterized. Many neuropeptides are very similar in their molecular structures, and often their receptors remain unidentified, thus, the ligand-receptor relationship of neuropeptide signalling is still largely unknown. Although some studies have shown that neuropeptides can regulate animal’s arousal level and behavioural states (Ardiel et al., 2017; de Bono & Bargmann, 1998; Flavell et al., 2013), few studies have mapped the facilitatory mechanism to the level of individual neuron resolution.

As seen in Chapter 2, the effect of tap-induced facilitation on the blue light ASH response lasted seconds to minutes and then dissipated gradually, therefore, I hypothesized that the facilitatory process underlying sensitization and dishabituation is mediated by neuromodulatory molecules. I started my search for neuromodulatory signalling components that are critical for sensitization of ASH response because the sensitization paradigm involved only one tap stimulus and one blue light pulse, allowing for a simpler experimental procedure. After identifying genes that altered sensitization, I tested strains of worms with mutations in these genes in the dishabituation paradigm to examine whether these genes altered dishabituation of the ASH response. These results would indicate whether sensitization and dishabituation are mediated by the same genetic and molecular components; that is, whether the two forms of behavioural plasticity are mediated by the same or different facilitatory processes.

3.2 Methods

3.2.1 C. elegans Strains

Two strains, AQ2755 and VG61 were used as genetic control groups in the experiments described in this chapter. AQ2755 worms express ChR2 exclusively in ASH, whereas VG61
worms express ChR2 strongly in ASH, but also weakly in two pairs of off-target neurons, ASI and PVQ.

Mutant alleles of flp-20(ok2964), frpr-3(ok3302), and frpr-3(gk240031) were crossed into AQ2755, and mutant alleles of pdf-1(tm1996), pdf-2(tm4393), pdf-r-1(ok3425), and gpc-1(pk298) were crossed into VG61 to make single or double mutant strains. In all experiments, mutants were tested together with the control strains they were crossed into, and comparisons were made between them.

### 3.2.2 Behavioural Paradigms

I used the standardized tap-induced sensitization and dishabituation paradigms in the experiments described in this chapter, and facilitation of the ASH reversal response was analyzed. For all groups, six plates of 40-80 worms were tested.

For sensitization, all worms were allowed to rest on the MWT for 300 s. At 300 s, the sensitized group received a tap stimulus, whereas in the naïve group, this tap was omitted. After a 10 s interval, a 2 s blue light pulse was delivered to photoactivate ASH in both groups, and the durations of the reversal responses to the light stimulus were measured for statistical comparison.

For dishabituation, all worms were given a 300 s period to acclimatize to the tracker. Starting at 300 s, 2 s light pulses were delivered every 10 s for 30 times to habituate the animals. In the dishabituated group, a tap stimulus was delivered 10 s after habituation training was completed, and in the spontaneous recovery group, no taps were delivered. After a 10 s interval, one 2 s blue light pulse was delivered in both groups, and initial, habituated, and facilitated (either by spontaneous recovery or dishabituation) reversal response durations to optogenetic stimulation of ASH were compared.
3.2.3 Data Analysis

For sensitization, a two-way ANOVA was performed to compare the response durations between naïve and sensitized groups, and across three different genotypes. If significant main effects were found, follow-up one-way ANOVAs with Tukey’s multiple comparisons post hoc test were performed, and if a significant interaction was found, an analysis for simple effects was performed.

For dishabituation, habituation in each strain was first verified by the observation that the final response level was significantly lower than the initial response level; this was done using a paired t test. Then, the durations of the final response to the test stimulus were compared between spontaneous recovery and dishabituation groups and across different genotypes using a two-way ANOVA. If significant main effects were found, follow-up one-way ANOVAs with Tukey’s multiple comparisons post hoc test were performed, and if a significant interaction was found, an analysis for simple effects was performed. For all statistical analysis, alpha was .05.

3.3 Results

3.3.1 Identifying Molecular Components Underlying Tap-Induced Sensitization

My first hypothesis was that neuromodulators were underlying the facilitatory process that leads to behavioural sensitization. Further, because the cross-modal sensitization effect appeared to be direction-specific, that a tap was capable of sensitizing the ASH response, but the converse was not true, I also hypothesized that the sensitization is mediated by neuromodulatory molecules released by the mechanosensory neurons. In the mechanosensory neurons in C. elegans three neuropeptide genes, flp-4, flp-8, and flp-20, are expressed (WormAtlas). A previous study from our lab found that FLP-20 peptides released from the touch receptor neurons
were critical for intermediate-term of memory for tap habituation after massed training (Li et al., 2013). Because flp-20 gene is involved in behavioural plasticity, I first investigated whether flp-20 was required for the behavioural sensitization of optogenetic ASH reversal response.

I tested control and flp-20 worms in the sensitization paradigm, and as seen in Figure 5, flp-20 mutants indeed showed a deficit in behavioural sensitization. A two-way ANOVA found a significant interaction between the genotypes of the worms and between the naïve and sensitized groups ($F(3, 47)=4.4$, $p=.009$). An analysis for simple effects was performed in naïve and sensitized conditions. It was found that there were no significant differences in the naïve response durations between the control and the flp-20 mutant ($F(3, 23)=.51$, $p=.677$). There was a significant difference in the sensitized response durations between different strains ($F(3, 23)=11.16$, $p<.001$), and multiple comparisons showed that the ASH response duration 10 s after a tap stimulus in flp-20 worms was significantly lower ($p<.001$). Thus, flp-20 appeared to be a key genetic factor underlying behavioural sensitization of the ASH reversal response.

The neuropeptides encoded by flp-20 are supposed to signal through yet unidentified G protein-coupled receptors (GPCRs), therefore, my next step was to identify the GPCRs through which FLP-20 peptides interact to exert their effect on behavioural sensitization. For an earlier study (Ardiel et al., 2017), I participated in performing a screen using RNA interference (RNAi) to individually knock down the expression of 57 GPCR genes and quantified their habituation and locomotion phenotypes. I independently analyzed the dataset and found several candidate genes that might alter sensitization, one of which was frpr-3. Knocking down frpr-3 using RNAi resulted in a normal baseline locomotion speed but a slower ASH reversal response speed, therefore, I hypothesized that frpr-3 might be one of the GPCR genes underlying behavioural sensitization.
When I tested *frpr*-3 mutant worms in the sensitization paradigm, I observed that mutations in this gene also led to a deficit in behavioural sensitization (Figure 5). I tested two loss-of-function *frpr*-3 mutant alleles (*ok3302* and *gk240031*), and in both cases, the *frpr*-3 mutants showed deficits in tap-induced facilitation in ASH response duration. Again, a significant interaction was found by a two-way ANOVA (*F*(3, 47)=4.4, *p*=.009), therefore, simple effects were looked for in the naïve and sensitized groups. The naïve response durations of two alleles of *frpr*-3 mutants were not significantly different from that of the control worms (*F*(3, 23)=.51, *p*=.677). There was a significant effect in the sensitization condition between worms of different genotypes (*F*(3, 47)=11.16, *p*<.001). Significant reductions of the durations of tap-sensitized response in both mutant alleles compared to control worms was confirmed by multiple comparisons (*ps*<.001). These results implicated the neuropeptide receptor FRPR-3 in behavioural sensitization of the ASH response.
Figure 5 Sensitization of the optogenetic ASH response is dependent on two neuropeptide genes. A tap stimulus sensitized the ASH response in the control group, and the response duration of the sensitized response was significantly higher than the naïve response duration. No differences were found in the naïve response duration between control and mutant worms. Worms with \textit{flp}-20 and \textit{frpr}-3(\textit{ok3302}) failed to show any facilitation in the ASH response duration compared to the naïve response level; another allele of \textit{frpr}-3(\textit{gk240031}) caused a significant reduction in the tap-induced facilitation of the ASH response. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
The behavioural data I collected for *flp-20* and *frpr-3* mutants suggested that FLP-20 neuropeptides and the neuropeptide receptor FRPR-3 interact with each other and together mediate the facilitatory process underlying tap-induced sensitization of ASH response; however, there was still a lack of direct evidence to demonstrate that they indeed function in the same signalling pathway. At the same time as I was doing these experiments, we discovered another lab was studying the role of *flp-20* in another tap-induced sensitization paradigm. As a result of our parallel findings, I then participated in a multi-lab collaboration and contributed to the project with our expertise in behavioural experiments. One of the collaborators performed an *in vitro* binding assay and showed that cells expressing *frpr-3* responded to exogenous FLP-20 peptides by increasing their intracellular Ca\(^{2+}\) transient (Chew et al., 2018). This confirmed that *flp-20* and *frpr-3* do directly interact as ligand and receptor. More importantly, the collaborative effort has pinpointed the RID interneuron in the worm as the major site of action: RNAi knockdown of *frpr-3* in only RID resulted in a deficit in the ASH response sensitization (Chew et al., 2018).

RID is a neuroendocrine cell that does not synthesize any canonical neurotransmitters. Instead, this neuron expresses a number of neuropeptide genes, including *pdf-1* and *pdf-2*. *Pdf-1* and *pdf-2* are homologs of *Drosophila* pigment-dispersing factor (PDF) in *C. elegans* (Frooninckx et al., 2012), and in previous studies PDF signalling has been implicated in regulating worm’s arousal or behavioural state (Barrios et al., 2012; Flavell et al., 2013). In our lab, we discovered a novel form of locomotion sensitization during habituation, and the locomotion sensitization was redundantly mediated by *pdf-1* and *pdf-2* (Ardiel et al., 2017). These data led to me to ask whether PDF signalling plays a role in the behavioural sensitization of the ASH reversal response.
I performed the sensitization experiment with PDF mutants. As shown in Figure 6, I found that pdf-1 was critical for the facilitatory effect. A two-way ANOVA found significant interaction, $F(5, 71)=2.39, p=.048$. A simple effect analysis was performed in the naïve and sensitized conditions. It was found that the durations of naïve response were not significantly different between all strains ($F(5, 35)=.65, p=.662$), but in the durations of tap-sensitized ASH response was significantly decreased in pdf-1 ($p=.023$) and pdf-1; pdf-2 ($p=.045$) mutants compared to the sensitized response duration in the control. Mutations in pdf-2 or pdf-r-1 did not affect the sensitized response duration. Thus, PDF-1 appeared to play an important role in the behavioural sensitization of ASH response.

Thus far, the pathway we have identified is that tap-induced sensitization enhances the response to photoactivation of ASH by causing the mechanosensory neurons to release FLP-20 peptides that bind to FRPR-3 receptor primarily in the neuroendocrine cell RID. RID releases a number of neuropeptides, one or more of which must bind to a GPCR on ASH. ASH expresses a number of GPCR genes, but almost all of these GPCRs have been identified as chemoreceptors, involved in odor sensation. Therefore, I focused on a G protein gamma subunit, GPC-1, to investigate whether it plays a role in the behavioural sensitization.

I tested gpc-1 mutant in the sensitization paradigm, and found that the sensitization was abolished in these worms (Figure 6). A two-way ANOVA was performed, and a significant interaction ($F(5, 71)=2.39, p=.048$) was found. Testing the simple effects revealed that no statistical differences were found in the naïve response durations between different strains ($F(5, 35)=.65, p=.662$). In the sensitized condition, one-way ANOVA ($F(5, 35)=2.83, p=.033$) and multiple comparisons revealed that in gpc-1 mutant, the duration of tap-sensitized ASH response was significantly lower than that in the control worms ($p=.046$). Worms with a mutation in gpc-1
showed a significant decrease in the tap-induced facilitation, suggesting that GPC-1 could be involved in the ASH response sensitization.
**Figure 6 Sensitization of the optogenetic ASH response is dependent on PDF-1 and GPC-1.** A tap stimulus sensitized the ASH response in the control group, and the response duration of the sensitized response was significantly higher than the naïve response duration. No differences were found in the naïve response duration between control and mutant worms. Mutant for *pdf-1* and double mutant for *pdf-1* and *pdf-2* failed to show any facilitation in the ASH response duration compared to the control, and PDF receptor mutant *pdfr-1* showed a tap-induced facilitation of the ASH response that is not statistically different from the control. *Pdf-2* mutant was sensitized by the tap stimulus, and the level of sensitized response duration was not significantly different from that in control animals. Mutation in *gpc-1* caused a deficit in sensitization that a tap was unable to sensitize the ASH response compared to control. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
3.3.2 Investigating the Differential Involvement of Genes in Tap-Induced Sensitization and Dishabituation

Having identified several genes critical for the behavioural sensitization of ASH response in the previous experiment, the next question I had was that whether the same genes would also be required for behavioural dishabituation of the ASH reversal response. If the genes playing a role in sensitization also play a role in dishabituation, it will suggest that the same facilitatory process is underlying both sensitization and dishabituation. Conversely, if genes differentially mediate sensitization and dishabituation, that mutations in these genes cause deficits in one form of facilitation but not in the other, then the results will suggest that the two facilitatory processes are dissociable.

I tested mutant *flp-20* and *frpr-3* worms in the dishabituation paradigm along with their genetic controls, to investigate whether genes affecting behavioural sensitization would also affect behavioural dishabituation. First, I confirmed that all control and mutant worms habituated to the blue light stimuli activating ASH to a final level that was significantly lower than the initial level (paired t test, $df=5$, $p<.05$ for all). Next, I compared durations of the response to the test stimulus 10 s after the tap stimulus in spontaneous recovery and dishabituated group. As shown in Figure 7, it appeared that back-to-baseline dishabituation was produced by a tap stimulus in the control animals, and the duration of the tap-dishabituated ASH response was longer than that of the spontaneously recovered response. Similarly, in *flp-20* and *frpr-3* mutants, tap-induced dishabituation was longer from the spontaneous recovery counterparts. I performed a two-way ANOVA and found that there was a significant main effect by the dishabituating stimulus ($F(1, 47)=21.02, p<.001$), but there were no significant effects between the strains ($F(3, 47)=1.7, p=.177$) nor a significant interaction ($F(3, 47)=.31, p=.819$). Closer examination with
two follow-up one-way ANOVAs revealed that, in both the spontaneous recovery ($F(3, 23)=1.58$, $p=.216$) and dishabituated groups ($F(3, 23)=.79$, $p=.512$), the response durations were not significantly different between different strains. Although $flp-20$ and $frpr-3$ mutations led to deficits in behavioural sensitization, they did not cause any deficits in behavioural dishabituation.
Figure 7 Dishabituation of the optogenetic ASH response is not dependent on two neuropeptide genes affecting sensitization. All groups habituated as the response duration decremented from the initial response level (upper two dashed lines vs lower two dashed lines; the two dashed lines represent mean+/−SEM of the initial and final response durations). A tap stimulus dishabituated the ASH response in the control group, and the response duration of the dishabituated response was significantly higher than the spontaneous recovery response duration. Mutant worms for flp-20 and frpr-3 (both alleles) all showed facilitation in the ASH response duration compared to their spontaneous recovery response level. There were no significant differences between strains in the spontaneously recovered or dishabituated response durations. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/− SEM, and asterisks indicate statistical significance.
I also tested PDF signalling mutants in the dishabituation paradigm. The results showed that PDFR-1 played a key role in behavioural dishabituation (Figure 8). A two-way ANOVA found significant main effects between strains ($F(11, 71)=4.03, p=.003$) and between spontaneous recovery and dishabituation groups ($F(1, 71)=18.21, p<.001$), but no significant interaction ($F(11, 71)=.23, p=.947$). None of the PDF mutants were statistically different from the control in the spontaneous recovery and dishabituated response groups as shown by two follow up one-way ANOVAs (for all multiple comparisons, $ps>.05$). Although none of the PDF genes appeared to play a statistically significant role in the tap-induced dishabituation of the ASH response, in pdf-2 and pdfr-1 mutants the difference between tap-dishabituated and spontaneously recovered responses appeared to be smaller than the difference in control group. Replications of this experiment will be required to determine whether pdf-2 or pdfr-1 are involved in the tap-induced dishabituation of ASH response.

 Pdf-1 was shown to be critical for the behavioural sensitization of ASH response, however, only a moderate effect of this gene was observed in behavioural dishabituation. The pdf-1; pdf-2 double mutant did not have a deficit in dishabituation, but the double mutation abolished sensitization of the ASH response.

 Last, I tested gpc-1 mutant in the dishabituation paradigm. A two-way ANOVA found no significant interaction, $F(11, 71)=.23, p=.947$, suggesting that tap-induced dishabituation in the control was not significantly different from dishabituation in gpc-1 mutant. Mutation in gpc-1 abolished the tap-induced sensitization of ASH response, but left the tap-induced dishabituation intact (Figure 8).
Figure 8 Dishabituation of the optogenetic ASH response is dependent on two neuropeptide genes that do not affect sensitization. All groups habituated as the response duration decremented from the initial response level (upper two dashed lines vs lower two dashed lines; the two dashed lines represent mean+/−SEM of the initial and final response durations). A tap stimulus dishabituated the ASH response in the control group, and the response duration of the dishabituated response was significantly higher than the spontaneous recovery response duration. Mutant worms for pdf-1, pdf-1 and pdf-2, and gpc-1 all showed facilitation in the ASH response duration compared to their spontaneous recovery response levels. Pdf-2 and pdfr-1 mutants showed no tap-induced dishabituation, and the dishabituated response was not statistically different from the spontaneously recovered response. For each bar, n=6 plates, and 40-80 worms per plate was tracked during the same time window on the same day. Error bars represent mean +/- SEM, and asterisks indicate statistical significance.
3.4 Discussion

In this chapter, I investigated whether genes could differentially mediate the response facilitation in sensitization and dishabituation by testing worms with mutations in a number of neuropeptide genes. My results clearly demonstrate that the two facilitatory processes, sensitization and dishabituation, are dissociable at a genetic level. Mutations abolishing sensitization left dishabituation largely intact (flp-20, frpr-3, pdf-1, and gpc-1), while no genes were clearly identified as critical components for dishabituation, it is possible that genes that did not affect sensitization could play a role in dishabituation (pdf-2 and pdfr-1). These results provided evidence that sensitization and dishabituation, two behavioural phenomena, have different genetic and molecular underpinnings, suggesting that they are two separate facilitatory processes.

In search of genes that affect tap-induced ASH response facilitation, I hypothesized that neuromodulators released by the mechanosensory neurons are required. My findings showed that FLP-20 peptides released by the touch receptor neurons were critical for the ASH response sensitization, and a GPCR neuropeptide receptor FRPR-3 was also a key component in the facilitatory process underlying the behavioural sensitization of ASH response. Through our collaboration, we confirmed the direct interaction between flp-20 and frpr-3, and a single neuroendocrine cell, RID, was identified as the key site of action of FRPR-3. Further, I found that a G protein gamma subunit, GPC-1, that is expressed in ASH neurons, led to a deficit in behavioural sensitization when mutated.

These data on the cell-specific effect of genes are particularly informative and useful to uncover the signalling pathway through which neuropeptides modulate neuronal excitability and behavioural facilitation. The molecular components can be mapped onto the fully annotated
connectome in *C. elegans*, and this will contribute to a better understanding of how behavioural plasticity is generated by the neural circuits consisting of individually identified neurons. Our collaboration proposed a model illustrating the signalling pathway from FLP-20 (Chew et al., 2018). In addition, my data suggest that GPC-1 is also involved (Figure 9). This narrows down the range of search for the GPCRs in ASH responsible for the tap-induced sensitization of ASH response. In fact, the non-chemoreceptor GPCRs expressed in the ASH have been identified, including receptors for monoamines (tyramine, octopamine, serotonin, and dopamine) and neuropeptides (NPR-1 and NTR-1). My preliminary results have ruled out the octopamine and tyramine receptors (Figure 9). Future experiments can examine whether mutants for candidate genes have the defective phenotype, and whether re-expressing the genes in specific cells can restore the behavioural deficit. These new data will fill in the gaps in the model to move us forward to piece together the complete molecular underpinnings of the facilitatory process.
Figure 9 A model of the facilitatory signalling pathway underlying the tap-induced behavioural sensitization of ASH response. The model illustrates how tap-activated release of FLP-20 peptides from mechanosensory neurons act through a neuropeptide receptor FRPR-3 primarily in the neuroendocrine cell RID to mediate the sensitization of ASH response. Additionally, a G protein gamma subunit, GPC-1, expressed in ASH is also involved in the sensitization of ASH response. A list of candidate neuromodulator GPCRs are shown as the possible upstream partner of GPC-1. A part of this model (from FLP-20 in touch receptor neurons to FRPR-3 in RID) is adapted from Chew et al. (2018).
My experiments demonstrated that genes can differentially mediate sensitization and dishabituation. Some mutant worms that did not sensitize could still be dishabituated by the very same tap stimulus. Conversely, other mutants that had no deficit in ASH response sensitization might have a reduction in the dishabituation of the ASH response. These observations provide strong evidence that sensitization and dishabituation are mediated by at least partially different molecular mechanisms, thus, the facilitatory processes underlying the two behavioural phenomena are dissociable.

The data on flp-20, frpr-3, pdf-1, and gpc-1 indicated a separation of the facilitatory processes, as these genes appeared to only play a role in the facilitatory process mediating the sensitization of ASH response, while dishabituation was not altered by mutations in them. The results on PDF signalling mutants were more intriguing. We previously discovered a novel form of sensitization of locomotion during habituation training, and pinpointed the role of PDFR-1 in mediating this locomotion sensitization (Ardiel et al., 2017). In this research, I found that pdfr-1 was not required for ASH response sensitization, as the pdfr-1 mutant sensitized to a similar level control. These data together with our previous findings suggest that there are multiple facilitatory processes that in parallel contribute to various forms of facilitation in different behavioural components. Also, PDF-1 appeared to be required for sensitization of ASH response, but the known PDF receptor PDFR-1 was not required; a strain with a double mutation in the PDF ligands and mutation in the receptor PDFR-1 produced opposite effects on sensitization and dishabituation. These observations point to the possibility that there are unidentified partners that PDF ligands and receptor interact with to exert their differential effects on sensitization and dishabituation of the ASH response.
One possible future experiment is to determine the site of action of PDF signalling in tap-induced facilitation. For a previous study (Ardiel et al., 2017), I helped to generate a number of transgenic strains that rescue PDF signalling components in different subsets of neurons and in muscles. I can test these worms in the sensitization and dishabituation paradigms to see whether the PDF-1-dependent sensitization and PDFR-1-dependent dishabituation is rescued with targeting specific subsets of neurons, in order to localize the site of action of PDF signalling.

Another possible future experiment is to explore whether other RID-released neuropeptides are responsible for the behavioural sensitization of ASH.

One potential limitation of my experiments is the differences in the genetic background of two controls strains. One of the control strains has off-target expression of ChR2 in neurons other than ASH. Although the expression level is low in off-target neurons, I could not rule out that these off-target neurons were also activated by blue light and participated in the behavioural changes. In Ardiel et al. (2016), we noticed that the two control strains habituated to ASH stimulation with different patterns, and my results in this chapter also showed that the naïve ASH response durations in two strains were different (~3 s in VG61 vs. ~1.5 s in AQ2235). However, for three of the genes I investigated, I have made strains using both transgenic backgrounds, and the effects of these genes in two different backgrounds largely agreed with each other (data not shown). For future experiments, I plan to generate mutant worms in a standard transgenic background that ChR2 is only expressed ASH to rule out the possibility that ASI and PVQ are responsible for the behavioural effect of tap-induced facilitation.
Chapter 4: General Discussion and Conclusions

In this thesis, my research was designed to answer the question of whether sensitization and dishabituation of a behavioural response are mediated by the same or by different facilitatory processes. My results showed that, in C. elegans, sensitization and dishabituation, two facilitatory processes in non-associative learning, can have different genetic and molecular underpinnings, and that the two facilitatory processes increasing animal’s behaviour on different state backgrounds can be dissociated at a genetic level.

Data in Chapter 2 demonstrated that the four components of the ASH reversal response, probability, duration, speed, and latency, could be sensitized or dishabituated by a tap stimulus to a greater or lesser extent and for differing durations. It is worth noting that while I attempted to design paradigms to produce sensitization and dishabituation so they could be studied in isolation, they are not two independent processes that always occur one their own. The two processes can be activated simultaneously, and depending on the organism’s current behavioural state and the property of the stimulus and paradigm, the behavioural effect is exhibited in the form of either sensitization or dishabituation or both.

Data in Chapter 3 identified a number of genes that are differentially involved in ASH response sensitization. For sensitization of the ASH response, the key site of action for some of the genes were localized to individual neurons. The neural underpinnings of dishabituation still have not emerged yet, however, my findings pointed to a plausible range of genes and cells to search and provided evidence to formulate new testable hypotheses, in order to eventually understand the cellular and molecular mechanisms of tap-induced dishabituation of the ChR2 activated ASH response.
This research adds to the existing body of knowledge in two perspectives: mapping out the neural circuit and molecular pathway(s) underlying sensitization of the ChR2-activated ASH response, and generating a novel interpretation of the Dual-Process Theory.

4.1 Identification of the Cellular and Molecular Pathways of Behaviour

This research produced behavioural data to validate the predicted involvement of genes in tap-induced sensitization of the ASH reversal response. The findings highlighted the power of *C. elegans* as a model system to understand the neural and molecular mechanisms of behaviour. With high-throughput behavioural tracking, optogenetics, candidate gene approaches, and cell-specific gene expression manipulation, our collaborative effort has identified a portion of the genetic components and localized them to a few neurons. With this promising approach, more can be done with the same logic and in the same fashion to complete the identification of the cellular and molecular pathways underlying sensitization of the ASH response.

The cellular and molecular mechanisms of dishabituation remain poorly understood and characterized. My research suggested that two neuropeptide gene, *pdf-2* and *pdfr-1*, could be potentially critical for dishabituation. I propose future experiments to search for the cellular basis of PDF signalling in ASH response dishabituation. By combining a candidate gene approach, cell-specific genetic manipulation, and *C. elegans* nervous system anatomy, it is reasonable to believe that we will identify the cellular and molecular mechanisms of tap-induced dishabituation of ASH response. The neural underpinnings of two facilitatory processes can be compared to each other to further scrutinize the relationship between the sensitization and dishabituation.
4.2 Reinterpretation of the Dual-Process Theory

The Dual-Process Theory was formulated largely based on behavioural observations before any molecular mechanisms underlying non-associative learning were elucidated. As such, the model was inevitably restricted by the scope of inspection and source of evidence at the time. An update of the classic Dual-Process Theory model is needed to more accurately describe, explain, and predict organism’s behaviour in non-associative learning.

As seen in Figure 2, while dishabituation was observed in response duration, above-baseline dishabituation, or sensitization was observed in response probability and speed. The tap stimulus in the dishabituation paradigm caused different components of a habituated ASH optogenetic response to increase to different levels. My data on the dissociable effects of genes also suggested that there are specific mechanisms mediating the sensitizing and dishabituating processes that do not overlap (Figures 5-8). Based on these findings, I proposed to enrich the content of the Dual-Process Theory with respect to simultaneously occurring sensitization and dishabituation. A stimulus can co-activate both sensitizing and dishabituating processes to produce facilitation of behaviour in the organism; the sensitizing process produces facilitation of naïve behaviour, whereas the dishabituating process specifically reverses habituation.

Also, in the original expression of the Dual-Process Theory, the “state system” was hypothesized to govern the amount of facilitation in the whole organism. This was contradicted in my data by the fact that facilitation of different response components in the same animals occurred to different levels and lasted different durations (Figures 1 and 2). If the whole organism is sensitized, so should be all the response components, and yet, I showed that is not always the case. In this research as well as in previous research, a large amount of data shows that neuromodulators, including neuropeptides, monoamines, and acetylcholine, play key roles in
mediating cellular, synaptic, and behavioural plasticity. Based on these observations, I hypothesized that the “state system” consists of a variety of neuromodulators, that can be released by sensory neurons and neuroendocrine interneurons in response to sensory events. The “state system” regulates the animal’s behavioural response through activating a number of shared and distinct neuromodulatory signalling cascades in the S-R pathways to alter behaviour. The pattern of neuromodulatory receptors shapes which components of the response will be affected by a given neuromodulator. Understanding the catecholeminome and the neuropeptidome will help deepen our understanding of the mechanisms by which experience shapes behaviour.
Figure 10 A revised Dual-Process Theory neuronal model of the non-associative pathways. A) The original model of the neuronal pathways underlying proposed by Groves and Thompson (model adapted from Thompson, 2009). Habituation process is hypothesized to specifically occur in the S-R pathway, whereas the “state system”, pictured as a neuronal pathway with synapses onto the S-R pathway. B) The revised Dual-Process Theory proposed based on the findings in this research. Rather than a defined neuronal pathway, I hypothesized that the “state system” is a variety of neuromodulatory molecules that are released upon sensory stimulation and differentially act on different components in the S-R pathway. The “state” resides in the composition and concentration of the neuromodulators in this pool.
4.3 Future Experiments

I have proposed several future experiments in each chapter, and specific considerations were given to the limitations of the experiments I performed, and the future direction of my investigation. In Chapter 2, I attempted to dissociate sensitization and dishabituation using different behavioural paradigms. One caveat is that behavioural responses may be mediated by multiple underlying processes, and some of the processes may have parallel effects on the same behavioural components, therefore it is challenging to differentiate which of these processes produce the behavioural changes. More careful designing of the paradigms may be better at capturing these facilitatory effects using behavioural paradigms. In Chapter 3, the candidate gene approach shows great potential, and can be further applied to investigate the unknown parts of the cellular and molecular pathways underlying the facilitatory processes, especially to identify novel genes that are critical for dishabituation.

In the investigations of the effects of a number of candidate genes on ASH response facilitation, I noticed a few genes that might cause a partial deficit with my short-interval paradigms (*pdf-1* and *pdf-2* in dishabituation; Figure 8). My question is whether there are multiple time-dependent facilitatory pathways that together mediate the increase in behaviour. By varying the interval between stimuli in the sensitization and dishabituation paradigms when testing mutant worms, it is possible to discover other molecular contributors to the behavioural facilitation in different phases, and unravel the complexity of the mechanisms underlying “simple” forms of learning.
4.4 Concluding remarks

In this thesis, we see that the simplest forms of learning do not have simple explanations – in fact, they are very complex. Two non-associative processes both facilitate animal’s behavioural response, but have different mechanisms. As researchers, we should always carry out detailed and controlled behavioural observations and consider alternative possibilities to make new discoveries.
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