The role of neuropeptides in learning: insights from *C. elegans*

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Abstract (150 words)

Learning is critical for survival as it provides the capacity to adapt to a changing environment. At the molecular and cellular level, learning leads to alterations within neural circuits that include synaptic rewiring and synaptic plasticity. These changes are mediated by signalling molecules known as neuromodulators. One such class of neuromodulators are neuropeptides, a diverse group of short peptides that primarily act through G protein-coupled receptors. There has been substantial progress in recent years on dissecting the role of neuropeptides in learning circuits using compact yet powerful invertebrate model systems. We will focus on insights gained using the nematode *Caenorhabditis elegans,* with its unparalleled genetic tractability, compact nervous system of ~300 neurons, high level of conservation with mammalian systems and amenability to a suite of behavioural analyses. Specifically, we will summarise recent

discoveries in *C. elegans* on the role of neuropeptides in non-associative and associative learning.

Keywords – *neuropeptides;* C. elegans*; associative learning; non-associative learning*

Introduction

Learning is a vital property that allows animals to adapt behaviour to a continuously changing environment. At a cellular level, learning leads to changes within neural circuits. These include rewiring of synaptic connections, alteration of the amount of presynaptically released neurotransmitters as well as changes in the number and sensitivity of postsynaptic receptors. Such processes are modulated by non-autonomous signals called neuromodulators [1]. In contrast to classical neurotransmitters released from synaptic vesicles, many neuromodulators are "extrasynaptically" released and transported across larger distances by volume transmission, generating auto-, para- or even endocrine effects.

One of the largest and most diverse groups of neuromodulators is represented by neuropeptides, short peptides (~3-100 amino acids) that primarily bind to the similarly diverse group of G protein-coupled receptors (GPCRs). Because of this diversity, neuropeptide-receptor signalling offers a rich versatility for the modulation of neural circuits [2,3]. Neuropeptide signals act on a range of spatiotemporal scales to shape neural circuit activity, from single neurons to a multitude of tissues, within tightly controlled timeframes to functioning throughout an organism's lifetime [4,5]. Indeed, neuropeptides play invaluable roles in shaping plastic behaviours, including learning and memory, across diverse species.

Despite substantial progress in illuminating neuropeptide functions in learning circuits, we are only beginning to understand the underlying mechanisms. Improved tools to study localisation, biochemical identity and function of peptides/receptors provide new opportunities to dissect peptidergic modes of action in learning circuits within a living animal. Indeed, invertebrate learning circuits have proven to be powerful models due to their compact size, genetic tractability and amenability to a sophisticated technological toolbox including automated behavioural analyses. In this regard, the nematode *Caenorhabditis elegans* stands out with a highly compact nervous system of ~300 neurons, for which the anatomical connectome (the network of synaptic connections) has been extensively described [6]. Here, we will summarise main insights into neuropeptidergic signalling in *C. elegans* learning. We will first briefly discuss new advances in understanding the conservation of neuropeptide systems, before elaborating on the role of neuropeptides in two categories of implicit memory: non-associative and associative learning

Conservation of Neuropeptide Signalling Systems

Neuropeptides are a diverse class of modulators present in all known metazoans [3,7]. However, their short amino acid sequence has posed a challenge in assessing neuropeptide phylogeny and evolutionary history. In addition, GPCRs display high degrees of mosaicism due to many duplication events that complicate our understanding of their classification [8]. Recently, long-range phylogenetic analyses have gained traction due to the increased availability of published genomic sequences, which improve the quality of large-scale analyses. This has been accelerated by the development of improved algorithms to mine these resources.

Besides neuropeptides, conserved neuropeptide receptors and neuropeptide processing machinery are found in all bilaterians, implicating an ancient evolutionary origin for neuropeptidergic signalling [3,7,9]. This has been corroborated by several independent large-scale phylogenetic studies. These studies showed that ~ 30 peptidergic signalling systems were present in the last common urbilaterian ancestor, before the divergence between protostome and deuterostome lineages [3,7].

The *C. elegans* genome encodes an estimated 150 neuropeptide GPCRs. It also contains at least 153 neuropeptide-encoding genes that give rise to over 300 predicted bioactive peptides, many of which have been biochemically confirmed [9-11]. The *C. elegans* genome encodes orthologs of many evolutionarily ancient neuropeptidergic systems [3,7]. These include orthologs of the oxytocin/vasopressin, gonadotropin-releasing hormone (GnRH), thyrotropin-releasing hormone (TRH), neuromedin U (NMU), tachykinin, myoinhibitory peptide (MIP), and neuropeptide Y (NPY) families [3,5,7,10,12,13]. For several neuropeptide systems, ligand-receptor interactions between orthologous neuropeptides and predicted GPCRs have been confirmed biochemically through receptor activation assays in cultured cells. For several of these, structural homology appears to correlate with functional similarity.

Neuropeptides in non-associative learning in C. elegans

Non-associative learning is the simplest form of learning and has been ubiquitously observed in animal species across evolution. Habituation and sensitization are two types of non-associative learning: habituation is the response decrement as a result of being repeatedly stimulated, whereas sensitization is the response facilitation produced by a novel and/or noxious stimulus. Habituation is conventionally framed as learning to ignore recurring stimuli

that do not provide biologically relevant information, while sensitization is framed as enhancing the robustness of responses to appetitive and aversive stimuli. Non-associative learning in *C. elegans* was first demonstrated by Rankin *et al.* in 1990 [14]. Since then, the field has generated a rich body of literature on the cellular and molecular mechanisms of learning in *C. elegans* (reviewed in [15]). Here, we will describe highlights from several recent studies implicating neuropeptide signalling pathways in aspects of non-associative learning.

The FMRFamide-related neuropeptides encoded by the *flp-20* gene have been shown to play key roles in both habituation and sensitization. Li et al. (2013) found that FLP-20 neuropeptides released from the mechanosensory touch receptor neurons (TRNs) were required for a massed training-induced, intermediate-term memory (lasting 12 hrs) for mechanosensory habituation. Interestingly, there is no evidence that FLP-20 is involved in either short-term or long-term habituation, as *flp-20* mutants formed normal short-term and long-term memories for habituation. These findings indicate that neuropeptides mediate specific phases of non-associative memory [16]. FLP-20 is also critical for sensitization. Chew et al. (2018) investigated the cellular and molecular pathways underlying mechanosensory-induced sensitization of an optogenetically elicited nociceptive response in the polymodal neuron ASH [17]. In this paradigm, mechanosensory stimulation enhanced the calcium transient in response to natural stimuli (0.5 M glycerol) in ASH, as well as the magnitude of the escape response (backwards movement/reversal) to optogenetic stimulation of ASH, and touch receptor neuron-released FLP-20 mediated this effect. Intriguingly, in these two reports FLP-20 mediates two opposite behavioural changes, response decrement and response facilitation. One possible explanation for this is that FLP-20 neuropeptides act on different cellular and/or molecular signalling components to mediate different types of non-associative learning. In intermediate-term mechanosensory habituation, the memory is correlated with a *flp*-20-dependent increase in the

presynaptic vesical density in the PLM mechanosensory neurons, presumably increasing neurotransmitter release by recruiting more synaptic vesicles to the presynaptic terminals [16]. On the other hand, mechanosensory-induced ASH sensitization requires FLP-20 signalling through the neuropeptide receptor FRPR-3, and one of its major sites of action, the neuroendocrine neuron RID, was identified [17]. Taken together, these studies show that the same neuropeptides released by the touch receptor neurons can produce both habituation and sensitization (**Figure 1**).

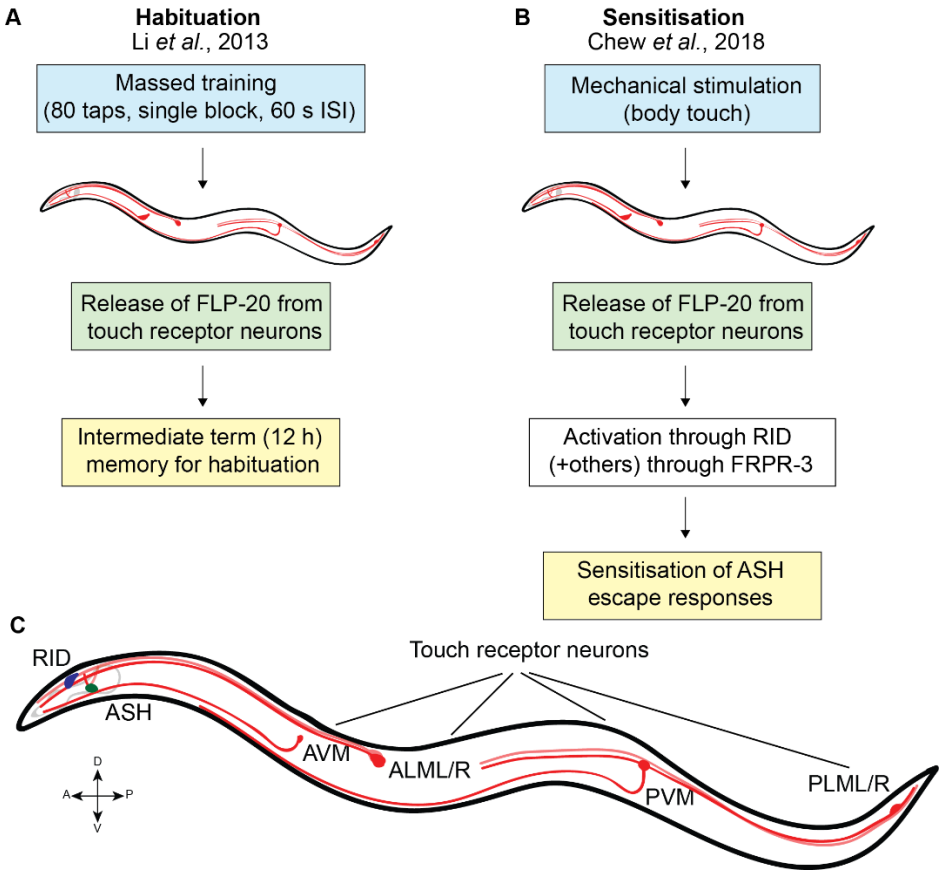

Figure 1: The neuropeptide FLP-20 is required for both habituation and sensitization. A) Intermediate-term memory for habituation in response to mechanical stimulation (taps) after massed training requires FLP-20 release from the touch receptor neurons (TRNs). ISI: Interstimulus interval. B) Sensitization of escape responses mediated by the nociceptive neuron

ASH requires FLP-20 release from the touch neurons, which act through the FRPR-3 receptor to activate the neuroendocrine cell RID. C) Schematic of specific neurons highlighted above: six TRNs (ALML/R, AVM, PVM, PLML/R) in the body and tail, the RID interneuron and ASH sensory neuron. For simplicity, only the soma of RID and ASH are shown.

The role of another class of neuropeptides, Pigment-Dispersing Factor (PDF) peptides, which are functional homologs of the vertebrate vasoactive intestinal peptide (VIP)[18,19], was revealed in studies of ASH-mediated nociceptive response habituation. In *C. elegans*, the *pdf-1* and *pdf-2* genes encode the two PDF ligands, and the *pdfr*-1 gene encodes the receptor. Repeated ASH optogenetic stimulation produces two sets of coordinated behavioural changes: habituation of the ASH-mediated escape response, and sensitization of forward locomotion [20]. These simultaneous changes may act together to trigger animals' dispersal away from where the nociceptive stimulus was received. The coordination of the two forms of plasticity is mediated by PDF signalling, as *pdf-1* and *pdf-2* mutants, as well as the *pdfr-1* receptor mutant, were defective in these coordinated behaviours. Interestingly, the sites of action of PDF signalling appear to be distributed across the nervous system and body wall muscle, as selectively re-expressing *pdfr-1* in either neurons or the muscle tissues in a *pdfr-1* mutant partially restored the behavioural effects. Thus, in this case, the PDF neuropeptides regulate the coordination between response habituation and ongoing locomotion sensitization, to adaptively shift the behavioural strategy to promote dispersal. These effects are summarised in Table 1.

Neuropeptides have also been shown to interact with other neuromodulators to mediate non-associative learning. *C. elegans* naturally avoid repulsive odours, for example, 2-nonanone, and the avoidance response to this volatile repellent can be sensitised if worms are pre-exposed

to the same odour [21]. Animals with mutations in genes encoding key components of the neuropeptide biosynthesis machinery, *egl-3* (proprotein convertase) and *egl-21* (carboxypeptidase N), failed to show this increased avoidance. Although neuropeptide synthesis is generally required for this learned avoidance response, it is unclear which specific neuropeptides are responsible. Interestingly, although dopamine is not involved in the sensitization, it is critical for maintaining the direction of avoidance away from the source of noxious stimuli. Taken together, neuropeptides and dopamine work together to increase the effectiveness of the learned avoidance response.

To summarise, the role of neuropeptides in non-associative learning is highlighted in three key findings: *(1)* Neuropeptides mediate different types of non-associative learning through different pathways, likely governed by the specific types of neuropeptide receptors expressed in different cells and tissues. *(2)* Neuropeptides are important for multi-component, coordinated behavioural changes in non-associative learning. *(3)* Neuropeptides interact with other neuromodulators to shape the response patterns in learning.

Neuropeptides in associative learning in C. elegans

Associative learning refers to processes where an association is formed between two stimuli. *C. elegans* has been used to study short-, intermediate- and long-term memory formation, as well as both positive and negative associative learning using a multitude of conditioned stimuli, including gustatory, olfactory, thermal, mechanical and gaseous cues, or more complex stimuli encoding a combination of multiple cues, such as the presence of pathogens or food. Several conserved neuropeptidergic systems have been shown to play a role in *C. elegans* associative learning, including homologs for insulin (INS-1) and insulin-like peptides (ILPs),

oxytocin/vasopressin (NTC-1), myoinhibitory peptide (MIP-1/NLP-38), neuromedin U (CAPA-1/NLP-44), elevenin (SNET-1), short neuropeptide F receptor signalling (NPR-1), and PDF-1 signaling (also mentioned above for non-associative learning) [4,5,10,12,22-28]. These effects are summarised in Table 1.

The specific effects of peptidergic signalling on associative learning often vary between different neuropeptides. While some *C. elegans* neuropeptides have been found to impact a wide diversity of learning behaviours, others have shown to modulate only specific associative learning circuits. For example, MIP signalling is required for gustatory aversive learning – to form a negative association between salt and the absence of food in the environment – but not for learning positive associations between the same salt cue and the presence of food [10]. In contrast, insulin-like signalling via the insulin receptor homolog DAF-2 appears to have an effect on both positive and negative associative learning [4,22,26,29]. Remarkably, insulin signalling has opposing effects on positive and negative associations: *daf-2* mutants fail to form a negative association when a conditioned stimulus is paired with starvation, whereas in positive associative learning paradigms (conditioned stimulus paired with appetitive cue), *daf-2* mutants show enhanced learning.

Neuropeptidergic signalling is not necessarily restricted to a specific conditioning cue either, as one neuropeptide can have modulatory effects on a variety of associative learning behaviours (**Figure 2A**) [4,23,30,31]. On the other hand, a specific learned behaviour can be modulated by a multitude of neuropeptides. For example, gustatory associative learning in *C. elegans* is modulated by at least four neuropeptides, including MIP-1, CAPA-1, NTC-1 and INS-1 (**Figure 2B**)[5,10,12,31]. At least five neuropeptides have also been shown to modulate learned

pathogen avoidance in response to environmental cues (INS-4, INS-16, INS-6, INS-7 and INS-11) [22,24-26].

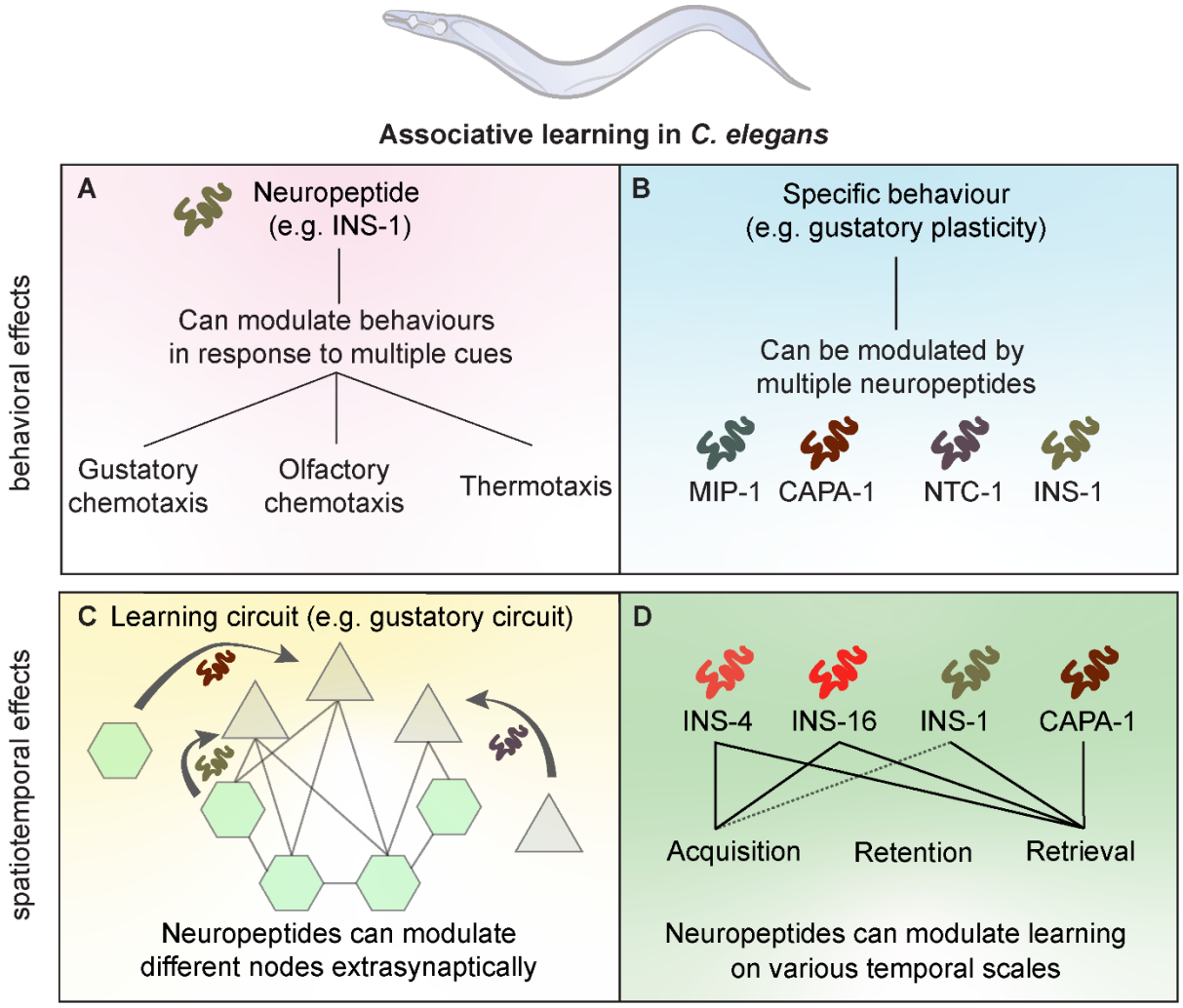

Figure 2: Studies of neuropeptide signalling in associative learning in *C. elegans* indicate that A) one neuropeptide can have modulatory effects on a variety of learned behaviours; B) a specific learned behaviour can be modulated by multiple neuropeptides; C) learning circuits are often modulated at different nodes by different neuropeptides; D) neuropeptides can differentially modulate specific stages of the learning process.

In such complex networks of neuropeptidergic modulation, it remains a challenge to unravel the individual effects of each of these modulatory systems on the learning circuit. Recent work reveals that the roles of individual neuropeptides in associative learning circuits can in part be

deduced by restricted spatiotemporal effects of neuropeptidergic signalling. Most neuropeptides have been found to act extrasynaptically [2]. This "wireless" mode of signalling can bypass restrictions in neural communication imposed by the anatomical connectome. Neuropeptides can thereby modulate diverse neurons in the learning circuit that do not have to be synaptically connected to the peptidergic cell (**Figure 2C**). Most examples of neuropeptidergic modulation in associative learning circuits so far illustrate modulation at the level of sensory neurons and interneurons. For example, INS-6 and INS-7 modulate pathogen avoidance learning by signalling between two pairs of sensory neurons that share no synaptic connections (ASI and URX) and a pair of interneurons (RIA) [6,22]. Likewise, NMU-like CAPA-1 neuropeptides signal extrasynaptically from sensory ASG neurons to another pair of sensory neurons (AFD) to modulate gustatory associative learning. When multiple neuropeptides modulate a specific learning circuit, such as in the case of gustatory aversive learning, different neuropeptide signals have been found to act on distinct nodes within the circuit (**Figure 2C**). For example, the neuropeptides INS-1, NTC-1 and CAPA-1 are all required for gustatory aversive learning, yet modulate distinct targets in the gustatory circuit, i.e. ASER, ASEL and AFD neurons [5,12,31]. Such concerted action of neuropeptides on different nodes in the learning circuit could be important to further tune learning behaviour to the animal's physiological needs [26]. INS-1 modulates gustatory aversive learning independently from other neuropeptides (*e.g.* MIP-1), indicating that different neuropeptides likely act in parallel to modulate learning[10].

Synaptic plasticity and altered neuronal activity are key cellular events during learning that are subject to neuropeptidergic regulation; however, the precise modulatory mechanisms are not completely understood. Indeed, it appears that the effects of neuropeptides on synaptic plasticity are not generalisable. For example, associative gustatory aversive learning, which

requires INS-1 signalling, increases calcium activity and decreases synaptic release from the ASER salt-sensory neuron [29]**.** In contrast, learning-induced changes in calcium activity of the ASE neurons are not affected by CAPA-1 neuropeptide signalling, even though *capa-1* is required for learning [5].

Besides targeted spatial effects, it has become clear that different neuropeptidergic pathways can modulate distinct temporal phases of *C. elegans* associative learning. Learning comprises at least three differentially modulated stages: memory acquisition, retention and retrieval; each of which can be separately modulated (**Figure 2D**). During olfactory associative learning in *C. elegans*, INS-1 signalling through DAF-2 is required at the retrieval stage, but INS-1 only partially contributes to acquisition of these memories [4,23]. Optogenetic manipulation of CAPA-1-expressing neurons in the gustatory associative learning circuit showed that CAPA-1 signalling is only required for the retrieval, but not the acquisition, of learned salt avoidance [5]. Importantly, the time window in which neuropeptides act differs between individual signals. In contrast to the stage-specific effects of CAPA-1 and INS-1, INS-4 and INS-16 are required in both acquisition and retrieval stages of learned pathogen avoidance behaviour [26].

Memory is traditionally also categorised as short-, intermediate- or long-term, each of which has specific molecular and behavioural hallmarks. Neuropeptides differentially affect aspects of short- and long-term associative memory formation in *C. elegans* [10,23]. For example, while MIP signalling was shown to similarly modulate short- and long-term gustatory associative memory, DAF-2 insulin-like receptor signalling differentially affects short- and long-term olfactory associative memory [10,23]. Investigations into the effects of single neuropeptides on both short- and long-term associative memory in *C. elegans* are limited to these examples.

However, several neuropeptide genes are upregulated after both appetitive and aversive long-term associative learning, suggesting a role in long-term memory processes [32,33]. In line with these findings, evidence from other vertebrate and invertebrate models suggests that differential neuropeptidergic modulation of short- and long-term memory may be a general feature in associative memory formation.

Table 1: Examples of neuropeptides and neuropeptide receptors modulating non-associative and associative learning in *C. elegans*. A question mark indicates neuropeptide systems for which the cellular focus remains unknown. Under "Effect", upward arrows (↑) denote neuropeptide systems that promote learning, where two upward arrows (↑↑) indicate neuropeptide systems for which loss-of-function results in abolished learning and a single upward arrow (↑) denotes a reduced ability to learn upon impaired peptidergic signalling. Downward arrows (↓) indicate suppressive roles for neuropeptidergic signalling in learning. TRN = touch receptor neuron. STM = short-term memory, LTM = long-term memory.

Neuropeptide gene	Neuropeptide receptor gene	Neurons (from → to)	Effect	Paradigm	Stage	Ref(s)
Non-associative learning						
flp-20	*frpr-3*	TRNs → RID	↑	mechanosensory-induced ASH sensitization	-	[17]
	?	TRNs	↑↑	intermediate-term tap habituation (massed training with 80 taps)	-	[16]
pdf-1; pdf-2	*pdfr-1*	?	↑	Integration of ASH response habituation and locomotion sensitization	-	[18]
Associative learning						
ntc-1	*ntr-1*	AVK → ASEL	↑	gustatory plasticity (NaCl + food absence)	-	[12]
mip-1	*sprr-2*	?	↑	gustatory plasticity (STM; NaCl + food absence)	-	[10]
		?	↑	salt avoidance learning (LTM; NaCl + starvation)	-	[10]
		?	none	salt concentration memory (NaCl + food)	-	[10]
capa-1	*nmur-1*	ASG → AFD	↑	gustatory plasticity (NaCl + food absence; NaCl + benzaldehyde)	retrieval (↑)	[5]
ins-1	*daf-2c*	AIA → ASER	↑↑	gustatory plasticity (NaCl + food absence)	-	[10,29,31]
	daf-2	?	↓	massed positive butanone association (STM; butanone + food)	-	[23]
		?	↓	spaced positive butanone association (LTM; butanone + food)	-	[23]
	daf-2	ASI & AIA →AWC	↑↑	benzaldehyde-starvation learning (benzaldehyde + starvation)	acquisition (↑); retrieval (↑↑)	[4]
		ASI & AIA →AWC	↑↑	butanone-starvation learning (butanone + starvation)	-	[4]
		ASI & AIA →AWC	none	butanone enhancement (butanone + food)	-	[4]
	daf-2	?	↓	positive temperature learning (temperature + food)	-	[30]

snet-1	-	ASI & ASK → ?	↓	benzaldehyde-starvation learning (benzaldehyde + starvation)	-	[27]
pdf-1	*pdfr-1*	MCM → ?	↓	gustatory plasticity (NaCl + food absence)	-	[28]
ins-4	*daf-2*	AWA → ?	↑↑	pathogen avoidance learning	acquisition (↑↑); retrieval (↑↑)	[26]
ins-16	*daf-2*	ADL → ?	↓↓	pathogen avoidance learning	acquisition (↓↓); retrieval (↓↓)	[26]
ins-6	*daf-2*	ASI → URX	↑↑	pathogen avoidance learning	-	[22]
ins-7	*daf-2*	URX → RIA	↓↓	pathogen avoidance learning	-	[22]
ins-11	*daf-2*	Intestine →ADF,ASI	↓	pathogen avoidance learning	-	[24]

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Concluding remarks

C. elegans is a highly effective model system in which to advance our understanding of the cellular and circuit basis of learning. Technical advances in genetics, optogenetics, and connectomics, as well as the potential for large-scale screens, provide a uniquely powerful approach through which we can investigate the molecular foundation of learning behaviour. Studying *C. elegans* learning behaviours thereby advanced our understanding of the pivotal and various roles by which neuropeptides affect nearly every aspect of non-associative and associative learning, on different spatiotemporal scales and interacting with a variety of pathways and signals. Together with its amenability to high-throughput *in vivo* behavioural and neurophysiological screening and new biochemical information on the interactions between neuropeptides and GPCRs [34], we expect that *C. elegans* will become an important model for further research in the context of learning and memory disorders.

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Figure legends:

Figure 1: The neuropeptide FLP-20 is required for both habituation and sensitization. A) Intermediate-term memory for habituation in response to mechanical stimulation (taps) after massed training requires FLP-20 release from the touch receptor neurons (TRNs). ISI: Inter-stimulus interval. B) Sensitization of escape responses mediated by the nociceptive neuron ASH requires FLP-20 release from the touch neurons, which act through the FRPR-3 receptor to activate the neuroendocrine cell RID. C) Schematic of specific neurons highlighted above: six TRNs (ALML/R, AVM, PVM, PLML/R) in the body and tail, the RID interneuron and ASH sensory neuron. For simplicity, only the soma of RID and ASH are shown.

Figure 2: Studies of neuropeptide signalling in associative learning in *C. elegans* indicate that A) one neuropeptide can have modulatory effects on a variety of learned behaviours; B) a specific learned behaviour can be modulated by multiple neuropeptides; C) learning circuits are often modulated at different nodes by different neuropeptides; D) neuropeptides can differentially modulate specific stages of the learning process.