CHAPTER 1

Neuroendocrine Regulation in the Genetic Model *C. elegans*

Charline Borghgraef[1*], *Pieter Van de Walle*[2*], *Sven Van Bael*[1], *Liliane Schoofs*[1], *Wouter De Haes*[1,2§], *and Isabel Beets*[1,3§]

[1]Functional Genomics and Proteomics, Department of Biology, KU Leuven, Leuven, Belgium
[2]Molecular and Functional Neurobiology, Department of Biology, KU Leuven, Leuven, Belgium
[3]Cell Biology Division, MRC Laboratory of Molecular Biology, Cambridge, UK

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1.1 A brief history on the model organism *C. elegans*

Research on the neurobiology of *Caenorhabditis elegans* has its roots in the 1960s, when Sydney Brenner proposed to use the nematode as a model organism for studying development and functioning of the nervous system. Brenner (Nobel Prize 2002) pioneered *C. elegans* genetics, by isolating and genetically mapping hundreds of mutant strains. Two decades later, John White and colleagues reconstructed the anatomy and synaptic connections (connectome) of all 302 *C. elegans* neurons in the adult hermaphrodite from electron micrographs. More recently, the wiring diagram of the posterior mating circuit in the adult male was mapped. Because *C. elegans* has a fixed number of somatic cells, researchers were able to construct a complete cell lineage by tracking the fate of each cell from fertilization to adulthood. This work was achieved by John Sulston and Robert Horvitz (Nobel Prize 2002), Judith Kimble, David Hirsh and Einhard Schierenberg. The neuronal connectome and cell lineage map allowed unprecedented insight into the worm's anatomy, development and neuronal makeup. These resources provided the basis for several key discoveries, including the characterization of genes regulating programmed cell death and axon guidance.

At the start of the genomic era in the 1990s, *C. elegans* was one of the simplest and best-studied animals available for undertaking whole-genome sequencing.

*,§These authors contributed equally

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The nematode was the first multicellular eukaryote to have its genome sequenced, a project completed in 1998. In the same year, RNA-interference (RNAi) was first demonstrated by Andrew Fire and Craig Mello (Nobel Prize 2006) using *C. elegans*. It has since been widely adopted as a tool for gene silencing in many organisms. *C. elegans'* transparent body facilitated another breakthrough that revolutionized the analysis of gene function. In 1994, Martin Chalfie (Nobel Prize 2008) showed that DNA encoding green fluorescent protein (GFP) could be used to mark gene expression *in vivo* in *C. elegans*. These landmark discoveries have been fundamental for establishing *C. elegans* as a versatile, genetic model system which is used today for studying questions on diverse research topics ranging from aging to metabolism, behavior, innate immunity and neuroendocrinology.

1.2 *C. elegans* genetics and anatomy

C. elegans is a small, free-living nematode that has two sexual forms: hermaphrodites and males (Figure 1.1). Both sexes have five autosomal chromosomes. Males have one X chromosome resulting from a spontaneous non-disjunction during meiosis, which occurs at low frequency (0.1%). After mating, the proportion of male progeny rises to 50%. Self-fertilizing hermaphrodites have two X chromosomes. They are easily cultivated and ensure transfer of homozygous mutations to the next generation. Therefore,

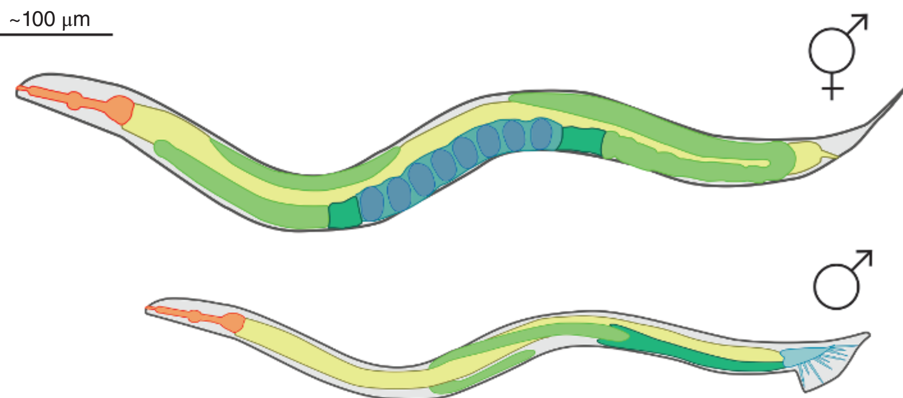

Figure 1.1 Schematic body plans of adult *C. elegans* hermaphrodite and male, showing the pharynx in orange, intestine in yellow, gonads in green and cuticle in grey. In hermaphrodites, the gonads are connected to the spermatheca (dark green), followed by the uterus with eggs (blue). Males have a single gonad, which is connected to the vas deferens (dark green) and male-specific copulatory apparatus (blue), consisting of a fanned tail with copulatory spicules.

they are studied far more commonly than males and used to maintain strain collections. *C. elegans* strains can be stored long-term by freezing them in a glycerol-rich solution at −80°C or in liquid nitrogen. The *C. elegans* research community has generated an extensive resource of mutants for most genes, which are summarized in the online database 'Wormbase', together with manually curated functional descriptions of all genes (www.wormbase.org). Over 21,000 protein-coding genes are annotated in the *C. elegans* genome (~100 Mb), over 30% of which have human orthologs.

Adult *C. elegans* have an invariant number of somatic cells (eutely). Adult hermaphrodites measure around 1 mm in length and consist of 959 somatic nuclei, including 302 neurons. The adult male comprises 1031 somatic nuclei with 381 neurons. Most male-specific neurons are located in the copulatory circuits of the male tail. Similar to other nematodes, *C. elegans* has a simple body plan (Figure 1.1) that consists of an unsegmented inner and outer tube, separated by the pseudocoelomic body cavity. The outer tube contains the cuticle, the hypodermis, the muscles, the neurons and the excretory system; the inner tube comprises the pharynx, the intestine and the gonads. The most important endocrine sites in *C. elegans* are the nervous system, the intestinal and the gonadal tissues.

The small nervous system of *C. elegans* and its fully mapped connectome make it a prime model for studying the neuroendocrine control of physiology and behavior. The *C. elegans* neural network consists of two distinct systems: the large somatic nervous system (282 neurons) and a smaller pharyngeal nervous system (20 neurons) (Figure 1.2). The pharyngeal nervous system drives pumping of the pharynx and operates largely autonomously. The majority of neurons in the somatic nervous system have cell bodies in the head. Their processes are organized in a nerve ring surrounding the pharynx. A smaller number of somatic neurons are located in the lateral and tail ganglia, with processes that often project into the nerve ring. Sensory perception primarily relies on two symmetrically placed multicellular sensory organs, called amphids, which are located in the head. They can detect a wide range of sensory cues including olfactory, mechanical and water-soluble chemical stimuli. Smaller sensory organs, termed phasmids, are laterally located in the tail and are involved in the integration of stimuli sensed at the anterior and posterior parts of the body. For example, the phasmid neurons PHA and PHB, together with the polymodal amphid neuron ASH, mediate behavioral responses to chemical repellants.

The worm's alimentary system – comprising the pharynx, intestine and anus – is involved in feeding and digestion. Since *C. elegans* consumes microorganisms, the intestine is also involved in immune and stress responses. In addition, the intestine and pharynx play important roles in the regulation of metabolic and endocrine processes, and in the storage of macromolecules. For example, the intestine is a main target site for insulin-like peptides. The

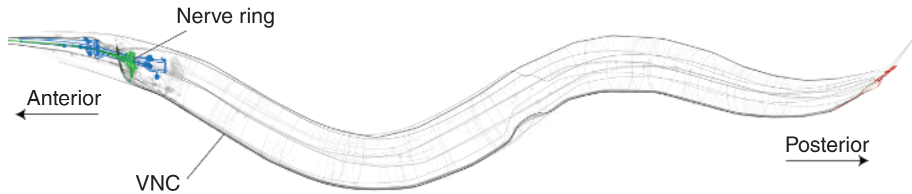

Figure 1.2 Schematic wiring diagram of the *C. elegans* hermaphrodite nervous system, which includes 20 pharyngeal neurons (blue) and 282 neurons of the somatic nervous system. Cell bodies of neurons in the somatic nervous system are primarily located in ganglia in the head and tail, and along the ventral nerve cord (VNC). Most head neurons are organized around a ring-shaped bundle of neuron processes, called the nerve ring. Over 60% of all somatic neurons project axons or processes into the nerve ring. The detection of sensory stimuli relies largely on the amphid neurons (green) in the head and phasmid neurons (red) in the tail.

somatic gonad also expresses several bioactive peptides and is thought to be the main site of synthesis of steroid hormones, termed dafachronic acids, which are involved in the regulation of development and lifespan. Males have only one gonadal arm for spermatogenesis (Figure 1.1). Hermaphrodites have two gonadal arms (Figure 1.1), in which oogenesis occurs in the distal tips. Hermaphrodite spermatogenesis takes place during development in the distal gonad, and sperm is stored in the spermatheca near the uterus.

1.3 *C. elegans* life-history

C. elegans is found worldwide, predominantly in humid and temperate environments. The nematode is commonly present in composting plant material, on plant stems, in rotting fruit and other bacteria-rich substrates. Its life-cycle consists of an embryonic stage, four larval stages (L1 to L4) and an adult stage. The timing of transitions between each stage depends on ambient temperature, but usually takes between three to four days from egg to adulthood (Figure 1.3). One of *C. elegans*' appealing features is its short generation time. The embryogenesis of hermaphrodites mainly occurs *ex utero* and lasts ~11 hours (at 20°C). The transition through the four larval stages typically requires ~65 hours. The end of each larval stage is characterized by a phase of lethargy and molting of the cuticle. In hermaphrodites, spermatogenesis takes place only during the fourth larval stage, after which oocytes are exclusively produced. Adults can lay eggs for up to 5 or 6 days and live for up to 3 weeks. *C. elegans* is easy to cultivate in the laboratory as the only requirements are nematode growth medium (NGM) agar plates seeded with *Escherichia coli* OP50 bacteria and a temperature-controlled incubator. Worms are typically grown at 20°C. Under these conditions, animals should be transferred to fresh plates every two to three days.

During larval development, several checkpoints exist that may cause *C. elegans* to enter states of arrested development and increased stress resistance. Transfer

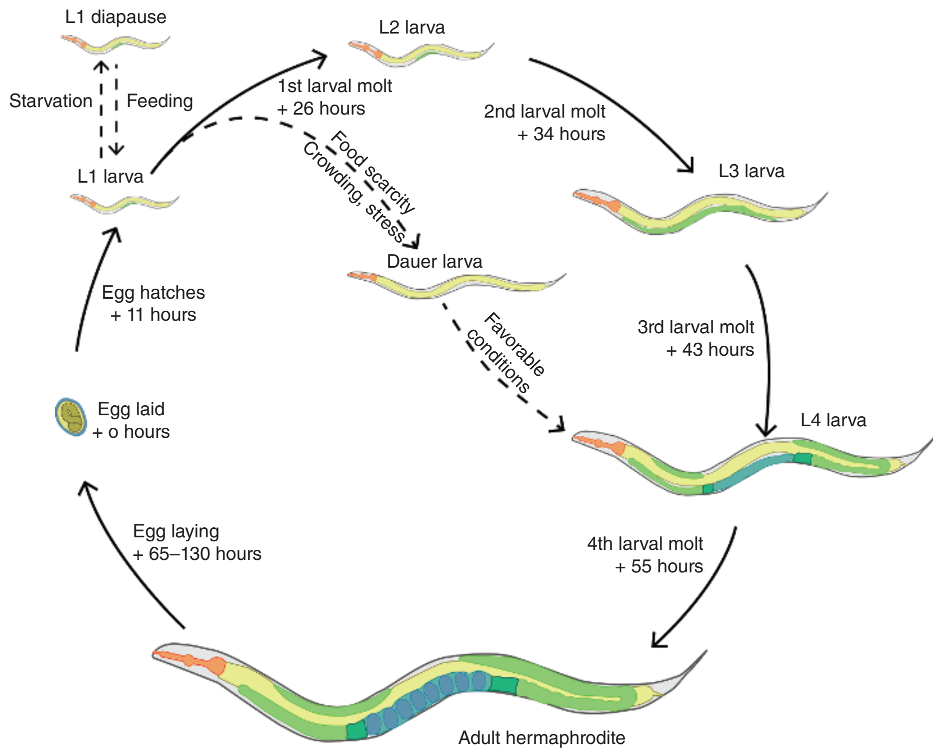

Figure 1.3 Lifecycle of *C. elegans* at 20°C. Adult hermaphrodites can lay eggs after self-fertilization or mating with a male. After 11-16 hours, the eggs hatch and develop into L1 larvae. These larvae can enter a reversible developmental arrest if starved. L1 develop subsequently into L2, L3, and L4 larvae, or go into another arrested developmental state termed 'dauer' during the first larval molt, when food is scarce, conditions are stressful, or the environment is crowded. Stress-resistant dauer larvae can rejoin the normal developmental cycle by molting into L4 larvae when conditions improve. L4 larvae molt once more into fertile adults. The entire development, from egg to adult, takes around 3 days.

into these arrested states is primarily controlled by the amphids and relies on neuroendocrine cascades, including insulin-like and transforming growth factor (TGF)-β-like signaling. If worms hatch in an environment that lacks food, they enter a state of altered metabolism, termed L1 diapause, in which they can survive for up to two weeks. L1 arrested worms resume their reproductive development when food is present. A second state of arrested development is an alternative third larval stage, termed the 'dauer stage', which can be induced by crowding, the lack of food, or the presence of other stressors in the environment (Figure 1.3). Dauer larvae are more resistant to stress and can live over four times longer than *C. elegans* adults. When conditions become favorable, the dauer larva resumes its molt into the L4 and adult stages. The dauer state is referred to as a 'non-aging state', as it does not affect the lifespan of *C. elegans* at the adult stage.

The lifespan of adult *C. elegans* is two to three weeks. Upon aging, adults display several morphological defects, some of which are reminiscent of human ageing. For example, muscle mass is lost and the cuticle becomes increasingly disorganized, leading to the formation of 'wrinkles' and loss of cuticular stability. Aging worms also shrink in size and show gradual decline in their ability to learn and retrieve memory. *C. elegans* is a prime model for the genetic study of aging, including cognitive decline which is regulated by insulin-like endocrine signaling.

1.4 Neuroendocrine signaling systems in *C. elegans*

1.4.1 Neuropeptides

C. elegans has a broad repertoire of neuropeptides that are typically derived from inactive precursor proteins, containing one or multiple neuropeptides. The *C. elegans* genome encodes four genes for proprotein convertases (PCs) that cleave peptides from their precursor (*kpc-1*, *egl-3*, *aex-5* and *bli-4*), all of which display homology to the Kex2/Subtilisin family of PCs in humans. After proteolysis, carboxypeptidases, which in *C. elegans* are encoded by *egl-21*, *cpd-1*, and *cpd-2* genes, catalyze the removal of paired basic amino acids at the cleavage site. Many neuropeptides require post-translational modifications that are essential for their biological activity and stability *in vivo*. These include C-terminal amidation, N-terminal conversion of glutamate to pyroglutamate, glycosylation, acetylation, sulfation and phosphorylation.

The *C. elegans* genome encodes at least 154 neuropeptide precursor genes that are classified in three families: the insulin-like (INS) peptides, the RFamide (FLP) peptides, and all other neuropeptide-like (NLP) proteins. The majority of neuropeptides, with the exception of the insulin-like peptides, are thought to signal via G protein-coupled receptors (GPCRs), of which more than 150 genes are predicted in the *C. elegans* genome.

FLPs are named for the presence of a C-terminal RFamide motif. In total, 31 *flp* genes (*flp-1* through *flp-28*, *flp-32*, *flp-33*, *flp-34*) have been annotated in *C. elegans*. They are involved in a variety of physiological and behavioral processes, such as locomotion, reproduction, sleep-like behavior, and learning.

Bioinformatic analyses have predicted 40 genes encoding insulin-like peptide precursors in *C. elegans*. INS-1 displays the highest degree of sequence and structural homology to human insulin. Other *C. elegans* INS peptides exhibit remarkably little sequence homology, and their discovery was primarily based on their structural similarity to mammalian insulin. All *C. elegans* INS peptides are thought to signal via DAF-2, a tyrosine kinase receptor homologous to the human insulin receptor. Activation of this receptor inhibits the relocation of the forkhead box O (FOXO) transcription factor DAF-16 to the nucleus, resulting in reduced transcription of downstream effector genes. The large number of insulin-like peptides in *C. elegans* and their expression in sensory

amphid neurons has led to the assumption that different insulin peptides act as inhibitors or activators of DAF-2, in order to integrate environmental cues during development. This includes the regulation of L1 diapause, dauer formation, stress tolerance and lifespan. Furthermore, INS-1 was found to be essential for starvation-associated learning and memory.

The NLP family of neuropeptides is highly diverse and groups all peptide sequences that are structurally unrelated to insulin or RFamide neuropeptides. So far, 83 neuropeptide-like genes have been described in *C. elegans* (*nlp-1* through *nlp-82*, and *snet-1*). These include homologs of human neuropeptide systems such as gonadotropin-releasing hormone (GnRH), vasopressin/oxytocin, cholecystokinin, thyrotropin-releasing hormone (TRH), and neuromedin U (NMU) signaling systems. Genetic studies revealed that these conserved neuropeptide systems exert functions in *C. elegans* similar to those of their counterparts in mammals. For example, a GnRH-like system regulates the onset of egg-laying in *C. elegans*, similar to GnRH-mediated control of sexual maturation during puberty. A *C. elegans* vasopressin/oxytocin-related neuropeptide pathway has a conserved role in associative learning and reproductive behaviors. The nematode's cholecystokinin homolog regulates fat content and digestive secretion as well as food-searching behavior. TRH signaling long remained elusive in protostomes, but a TRH-related system was recently discovered in *C. elegans*. It promotes postembryonic growth, similar to the function of human TRH.

1.4.2 Biogenic amines

Because of their function as neurotransmitters or neuromodulators, biogenic amines are essential for many biological processes such as development, neuronal signaling and plasticity. The main biogenic amines in humans include dopamine, norepinephrine, epinephrine, histamine, serotonin and melatonin. *C. elegans* synthesizes dopamine, serotonin and melatonin, but seems to lack adrenergic and histamine signaling. Like most invertebrates, *C. elegans* also produces octopamine and its precursor tyramine. Octopamine has structural similarities to norepinephrine and performs functions similar to adrenergic signaling in vertebrates, suggesting that octopamine is an invertebrate counterpart of norepinephrine. However, norepinephrine and octopamine signaling were recently shown to coexist in several marine invertebrates, indicating that octopamine and adrenergic signaling already evolved in a common ancestor of bilaterian animals. Over the years, *C. elegans* has proven to be a good model system for studying biogenic amine signaling. For instance, cell death of dopaminergic neurons affects dopamine signaling and has been shown to underlie motor deficiencies associated with Parkinson's disease. As proteins involved in dopamine signaling are well conserved between *C. elegans* and humans, the nematode has been used as a model for several dopamine-related disorders including Parkinson's.

1.4.3 Lipid-derived neuroendocrine signals

While *C. elegans*' neuropeptidergic systems and amines have been well studied, much less is known about its lipidergic endocrine systems. The main lipid-derived signaling molecules in *C. elegans* are the steroid-like dafachronic acids (DAs) and the ascaroside class of glycosides.

DAs are bile-acid-like steroids that are mainly involved in the regulation of development and adult lifespan. The two primary bioactive DAs, Δ4- and Δ7-DA, are generated by the cytochrome P450 DAF-9 from cholesterol derivatives. These molecules bind to the nuclear hormone receptor DAF-12, triggering a variety of responses. During development, steroid signaling is central in the decision between normal development and dauer formation. When conditions are optimal, insulin and TGF-β signals are secreted from the amphid neurons, resulting in the production of bioactive DAs, which activate DAF-12 to stimulate normal development. Experiments using *daf-9* deletion mutants have implicated DAs in the control of adult lifespan, i.e. in the lifespan extension following germline ablation. As DAF-9 is expressed in the gonad, it is thought to be one of the prime sites of DA synthesis, although experimental evidence for its production is lacking. Other potential sites for DA synthesis – based on expression of DAF-9 – are the hypodermis and spermatheca.

The *C. elegans* ascarosides are likewise involved in the regulation of development, as well as in male attraction behavior. Ascarosides consist of an ascarylose sugar linked to an aliphatic side chain and function primarily as excreted pheromones. In the context of development, several *C. elegans* ascarosides are also termed 'dauer pheromones', due to the fact that high concentrations of ascarosides in the environment are a signal for overcrowding and can trigger dauer formation.

1.5 Characterization of neuroendocrine signaling components in *C. elegans*

1.5.1 Omics tools for identifying neuroendocrine factors

Techniques for profiling metabolic, transcriptional and protein fingerprints (-omics tools) are well established in *C. elegans* and have been invaluable for determining protein- and lipid-derived neuroendocrine signals in this model system. These tools include metabolomics for the study of amines or lipid-based components (Butcher, 2017), and proteomics for (differential) profiling of larger protein molecules, such as TGF-β. An off-shoot of proteomics, peptidomics allows identification and quantification of neuropeptides. One of the most frequently used methods is liquid chromatography-mass spectrometry (LC-MS), which provides the opportunity to characterize all neuropeptides present in an organism, an organ or tissue, and has enabled identifying the neuropeptide complement of *C. elegans* (De Haes et al., 2015; Husson et al.,

2009). Using peptidomics, new neuropeptides can be identified by MS/MS de novo sequencing, or the peptide profiles of two (or more) conditions can be compared in a differential approach (De Haes et al., 2015). Cleanup of *C. elegans* peptide extracts for LC-MS analysis often includes delipidation and enrichment of the neuropeptide mass fraction. In addition, solid phase extraction proves to be useful to increase peptide identifications (De Haes et al., 2015).

1.5.2 Reverse pharmacology

The discovery of ligand-receptor couples is a crucial step in unraveling neuroendocrine signaling pathways. Many ligand-receptor interactions in *C. elegans* have been characterized by reverse pharmacology. In this strategy, a receptor of interest is expressed in a heterologous system, usually in cell cultures, and screened with a library of putative ligands.

Many neuroendocrine signals, such as neuropeptides and amines, activate specific GPCRs. Ligands can be identified by co-expressing the receptor with promiscuous or chimeric G proteins and monitoring their activation through changes in the concentration of secondary messengers (e.g. β-arrestin, cAMP or calcium). One of the most frequently used tests is the aequorin-based calcium mobilization assay. Apoaequorine, together with its cofactor coelenterazine, forms the calcium-sensitive photoprotein aequorin. The assay requires a recombinant eukaryotic expression system, such as Chinese Hamster Ovary (CHO) cells, in which a mitochondrially targeted apoaequorin and a promiscuous Gα protein, such as human $G\alpha_{16}$, are co-expressed. The receptor of interest is expressed in this cellular system, usually by transient transfection. Then, the cells are loaded with coelenterazine and screened with a compound library. Activation of the receptor causes an increase in intracellular calcium. This increase is facilitated by the promiscuous $G\alpha_{16}$, which has the ability to direct intracellular signaling of most GPCRs to a calcium flux, regardless of the endogenous G protein coupling of the receptor. The rise in calcium levels activates aequorin through its calcium binding sites, which induces the oxidation of coelenterazine and emission of blue light. The light's intensity is proportional to the level of receptor activation (Van Sinay et al., 2017).

1.5.3 The *C. elegans* genetic toolbox

One of *C. elegans*' main benefits as a model for addressing questions in neuroendocrinology is its genetic tractability and short generation time. Many techniques for generating mutant strains, including targeted genome-editing tools, are established in this model system. Because hermaphrodites are self-fertilizing, mutant strains can be easily maintained.

C. elegans mutant strains have historically been generated by random mutagenesis, most often using chemical mutagens such as ethylmethanesulfonate (EMS) or trimethylpsoralin (TMP) followed by ultraviolet light (UV) activation (UV/TMP). Many of the *C. elegans* knockout strains provided by the

Caenorhabditis Genetics Center (CGC) or by the National BioResource Project (NBRP) were created using these techniques. As the mutagenesis treatment also induces background mutations, mutant strains should be backcrossed to a wild-type background before using them in phenotypic studies.

Recently, gene-editing tools such as CRISPR/Cas9, TALENs and Zinc Finger Nucleases have been introduced, which allow generating targeted mutations in a gene of interest. These tools also provide several advantages for unraveling neuroendocrine signaling pathways. For example, CRISPR/Cas9-mediated knockout of the TRH-related *trh-1* gene in *C. elegans* allowed establishing its conserved role in the regulation of postembryonic growth (Van Sinay et al., 2017). Besides inducing gene-specific mutations, gene-editing techniques can be used to generate conditional mutants or reporter knock-in strains to study the effect or localization of neuroendocrine signaling components in specific cells or tissues. In *C. elegans*, protocols for diverse applications have been optimized [reviewed in (Chen et al., 2016; Dickinson and Goldstein, 2016)].

Besides gene knockout, RNA interference (RNAi) is often used for reverse genetics in *C. elegans*. Double-stranded RNA (dsRNA) can be introduced by several methods, which result in systemic RNAi: 1) Microinjection of dsRNA or sense and antisense RNA (sas) encoding transgenes is highly efficient, but is labor-intensive and less suited for high throughput experiments. These drawbacks can be overcome by delivering dsRNA through 2) feeding or 3) soaking. The dsRNA is taken up by the gut cells and subsequently distributed to other cells. The versatility of RNAi tools in *C. elegans* provides efficient ways of knocking down gene expression, allows study of lethal genes for which no mutants exist, and offers the possibility to temporally knock down genes as well as carry out high throughput reverse genetic screens (JoVE Science Education Database, 2017). For example, RNAi was used to knock down the *C. elegans* ortholog of the gonadotropin-releasing hormone (GnRH) receptor and discover its function in egg-laying behavior (Schoofs et al., 2009), illustrating the capacity of RNAi for neuroendocrine research.

Microinjection of transgenes is an indispensable genetic technique to study gene functions in *C. elegans*. Fluorescent reporter transgenes can be used to localize expression of a gene of interest, while promoter-gene fusions allow introducing expression of a gene in a cell-specific manner. Many state-of-the-art techniques such as calcium imaging and optogenetics rely on microinjection for the expression of transgenes. The construct of interest is typically injected into the cytoplasm of the syncytial gonads, after which it is incorporated into the nuclei of the oocytes. The injected constructs form multi-copy extrachromosomal DNA arrays that can be passed onto a part of the progeny, generating semi-stable transgenic strains. Following treatment with UV or γ-irradiation, the extrachromosomal array can be integrated into the genome. As this method results in random integration and potentially random mutation, the integrated strain needs to be backcrossed. As an alternative to microinjection, particle bombardment and single-copy MosSCI insertion can be used for the generation

of integrated transgenic strains (Frøkjær-Jensen et al., 2008; Hochbaum et al., 2010).

1.6 Neuroendocrine-regulated behaviors in *C. elegans*

Despite its small nervous system, *C. elegans* displays a broad range of behaviors that can often be automatically quantified. The nematode's genetic tractability and neural connectome facilitates unraveling the molecular basis and neural circuits that underpin behaviors. Synchronized populations of animals can be generated to standardize behavioral assays, for example, by picking animals of a selected stage onto a fresh growth plate, or by allowing adult worms to lay eggs for a 4- to 6-hour time period. When larger population sizes are required, synchronization can be achieved by collecting eggs from gravid adult animals using a bleach solution. *C. elegans* behaviors can be divided into several categories, which are further discussed here: 1) feeding, 2) egg-laying, 3) mating, 4) mechanosensation, 5) chemosensation, 6) thermal responses and 7) learning and memory (Hart, 2006).

1.6.1 Feeding

C. elegans employs a series of behavioral strategies to search for and differentiate between foods of differing quality. When feeding, *C. elegans* alternates between two behavioral states: roaming and dwelling. Roaming worms move swiftly and turn less to travel across a large distance. Dwelling is characterized by slower movement and increased turning. While roaming facilitates exploration, dwelling allows exploiting local food. When *C. elegans* is removed from food, it dwells for a short time, before entering a chronic roaming state. These food-searching strategies mainly rely on the stimulation and inhibition of interneurons. Four interneurons (AIA, AIB, AIY and AIZ) control movement via inhibition or stimulation of motor neurons, and are modulated by different dietary states. This modulation involves a vast neuroendocrine response, including cholecystokinin signaling and signaling to the GPCR NPR-9 via unknown ligands (Campbell et al., 2015).

In addition to roaming and dwelling, *C. elegans* shows feeding-related quiescence. After feeding, worms can enter a state of quiescence in which movement and food intake is strongly reduced, a state that is reminiscent of satiety quiescence in humans. The satiety state occurs even more prominently when worms are reintroduced to food after fasting. The mechanisms that control satiety-induced quiescence are distinct from those that control the choice between roaming and dwelling. Satiety-induced quiescence primarily relies on nutritional status signaled by the chemosensory and gustatory ASI neurons in the amphids through insulin and TGF-β pathways (Gallagher et al., 2013).

1.6.2 Egg-laying

In *C. elegans*, neuroendocrine signaling tightly controls reproductive behavior. A simple experimental set-up for studying hermaphrodite reproduction is an egg-laying assay. In such an experiment, synchronized adult worms are transferred to a new plate every few hours during their reproductive phase. The number of offspring produced during each interval is counted, revealing the temporal dynamics of egg-laying as well as the total brood size. Members of all major *C. elegans* neuropeptide families have been implicated in the regulation of egg-laying behaviors. Egg-laying also requires serotonin that is secreted from hermaphrodite-specific neurons (HSNs). The activity of these neurons is regulated by the RFamide neuropeptides FLP-10 and FLP-17 through the GPCR EGL-6. These and several other FLPs have been shown to directly or indirectly affect egg-laying by regulating the motor neurons of the egg-laying circuit and their synaptic input (Banerjee et al., 2017). Mutants for pigment dispersing factor (PDF) neuropeptides and their receptor show a delayed onset of egg-laying, but not of total progeny count (Meelkop et al., 2012). *C. elegans* insulin-like peptides are also involved in reproduction, likely through a broader effect of insulin signaling on development as a whole. Mutation of the insulin/insulin-like growth factor receptor DAF-2 is a well-known lifespan-promoting intervention in *C. elegans*, but also influences hermaphrodite reproduction, extending the reproductive span (from 5 days to 9 days) and delaying the decline in oocyte quality (Luo et al., 2010). The variety of neuropeptide signals involved in regulating hermaphrodite reproduction highlight the complex and intertwined nature of these signaling networks.

1.6.3 Mating

Male *C. elegans* display a number of sex-specific mating behaviors that are controlled by neuropeptides and other neuroendocrine factors. Under normal conditions, males locate a mate, scan the surface of the hermaphrodite body with their copulation apparatus in search of the vulva, and initiate copulation. Nematocin, a vasopressin/oxytocin-like neuropeptide in *C. elegans,* regulates male mating behavior. Its expression pattern is sexually dimorphic, showing activity in male-specific motor neurons and in the male copulation apparatus, aside from a number of neurons common to both sexes. Male animals deficient in nematocin fail to coordinate different steps of the mating behavior (Garrison et al., 2012). Mutants defective in neuropeptide signaling by FLP-8, FLP-10, FLP-12 and FLP-20 peptides also show aberrant male mating behavior (Liu et al., 2007).

1.6.4 Mechanosensation

C. elegans has the ability to sense a wide variety of stimuli, including mechanical cues. Depending on the type of mechanical stimulus, different mechanosensory neurons in the nose, the tail, and along the body are activated. A mechanical

stimulus can elicit a change in ionic current by two main types of proteins, i.e. TRP channels and DEG/ENaC channels. The main mechanosensory neurons are PLM, ALM and AVM (Bozorgmehr et al., 2013; Hart, 2006).

A gentle touch elicits a forward or backward movement dependent on the activated neurons. For example, a light touch on the nose will activate the ASH, OLQ and FLP sensory neurons and induce backward crawling – called the 'nose touch response'. Surprisingly, this response is governed by polymodal sensory neurons rather than specific mechanosensory neurons. Gentle touch to the anterior part of the body activates mechanosensory head neurons (ALM and AVM), which elicit a backward movement upon activation. Stimulation of the tail PLM neurons results in a forward movement (Bozorgmehr et al., 2013).

Tapping the culture plate typically causes worms to reverse their movement. Such mechanical stimulus evokes a vibration, which simultaneously activates head and tail mechanosensory circuits. The resulting backward movement is due to an imbalance in forward- (tail: two neurons) and backward- (head: three neurons) reacting neurons. Only adult worms exhibit reversals, as the AVM neuron is not yet properly connected in younger worms (Bozorgmehr et al., 2013; Sugi et al., 2014). Along with the previously described mechanosensory neurons, other neurons are involved in the tap withdrawal response. Four pairs of interneurons (AVD, AVA, AVB and PVC) integrate the information towards the motor neurons. In addition, PVD and DVA are paramount for forward and backward movement and integrate both forward and backward responses (Bozorgmehr et al., 2013; Sugi et al., 2014).

Neuronal glutamate is thought to be the main signaling molecule involved in the regulation of the nose touch circuit. Mutants for the glutamate receptor *glr-1* have a reduced response to nose touch, which is suppressed by a mutation in the PC *egl-3*. This finding suggests that peptidergic signaling inhibits nose touch responses in the absence of glutamatergic signaling (Campbell et al., 2015). Differences in touch sensitivity can be indicative of physiological changes, such as increased cuticle thickness, of altered neuroendocrine signaling (Herndon et al., 2002), or can imply changes in the mechanosensory transduction machinery.

1.6.5 Chemosensation

C. elegans' chemosensory system enables the detection of water-soluble and volatile compounds. It includes four main types of chemosensory organs: The two amphids are located in the head region and consist of 11 paired neurons (ADL, ADF, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB, and AWC). The phasmids are located at the posterior end and comprise PHA and PHB. In addition, the inner and outer labial pores surround the mouth of the worm and consist of chemosensory neurons (IL1, IL2, OLL and OLQ). All of these chemosensory neurons are directly or indirectly connected to the environment, and allow chemosensation of several stimuli (Menini, 2010). Salt sensation is one of the frequently used chemosensory responses in behavioral assays.

C. elegans is naturally attracted towards low concentrations of sodium chloride and other inorganic salts. This behavior is mainly governed by the ASE taste neurons. High concentrations of salt evoke osmotic avoidance, which is primarily mediated by the nociceptive ASH neurons. These neurons detect water-soluble compounds with high osmotic strength, such as high sodium chloride concentrations or fructose. Salt attraction and avoidance behaviors are influenced by neuropeptide signaling, which modulates neuronal and non-neuronal target cells (Beets et al., 2013; Hukema et al., 2006; Leinwand and Chalasani, 2013; Oda et al., 2011).

C. elegans can also sense volatile compounds. For this purpose, chemosensory neurons including AWA, AWB and AWC are of major importance. As most of the sensed odors arise from bacterial metabolism, they are often attractive. Similar to neurons involved in chemical sensing, olfactory neurons are each linked to a particular behavioral response. AWA and AWC mainly drive attraction, whereas AWB is linked to repulsion. Olfactory assays often use butanone, diacetyl or benzaldehyde as olfactory cues (Bargmann, 2006).

1.6.6 Thermosensation

Thermosensation and thermotaxis behaviors allow *C.* elegans to migrate towards preferred temperatures. Only a limited number of neurons with the ability to sense ambient temperature have been identified. AFD neurons are the main thermosensory neurons with specialized sensory endings. Beside their ability to sense temperature, thermal information is stored and processed in these neurons. Next, the processed information is transmitted to AIY interneurons by glutamate, which signals to motor neurons through RIA. AFD-ablated animals still exhibit the ability to migrate towards colder regions suggesting a role for other neurons in thermotaxis behaviors (e.g. AWC). *C. elegans* can also link thermosensation with cultivation state; hence, the ability to learn and remember a previously favorable temperature can be assessed as well (Aoki and Mori, 2015).

1.6.7 Learning and memory

Many of *C. elegans'* behaviors are subject to experience-dependent modulation. The nematode's behavioral responses depend on the external and internal environment, and can vary with previous experience or age (Ardiel and Rankin, 2010; Podshivalova et al., 2017). *C. elegans* shows various types of learning and memory, including short-term, intermediate-term and long-term memory. The ability to learn and store memory declines in ageing worms, which is influenced by insulin signaling (Kauffman et al., 2010; Li et al., 2016). Both associative and non-associative types of learning have been described and are controlled via neuroendocrine mechanisms (Ardiel and Rankin, 2010; Hoshiba et al., 2017; Menzel and Benjamin, 2013). For example, gustatory associative learning requires vasopressin/oxytocin-like and serotonin signaling, among

other pathways (Beets et al., 2012; Hukema et al., 2006). Here, we briefly highlight a few examples of each learning type.

1.6.7.1 Non-associative learning

Non-associative learning can be observed when *C. elegans* changes its behavior in response to a single stimulus. This includes chemo- and mechanosensory habituation, in which worms gradually ignore a repetitive stimulus to which they would normally react. Many non-associative learning paradigms exist for *C. elegans*, but a classic example is tap habituation (Figure 1.4). In this assay, an agar plate containing one or more worms is repeatedly tapped, resulting in backward movement. As the number of taps increases, the reversal probability and magnitude of the reversal response decreases. Differences in these parameters can be indicative of a role in habituation-driven learning and memory

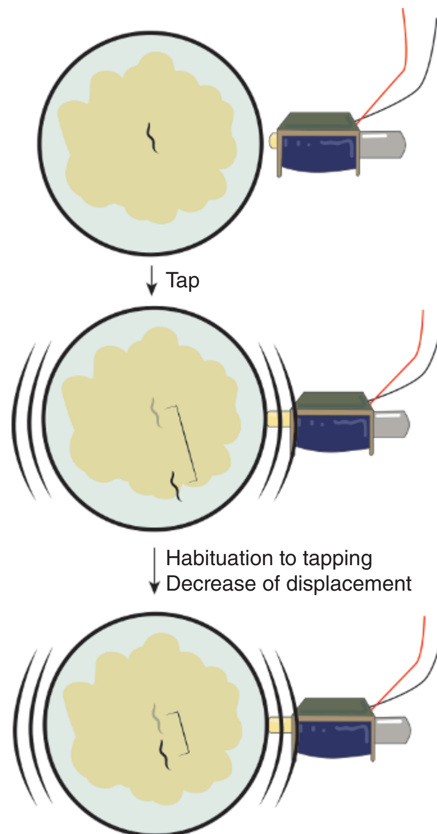

Figure 1.4 Tap habituation assay, in which habituation to a repeated mechanical stimulus is measured. An automatic tapping device applies the mechanical stimulus at a constant interval, resulting in backwards motion of the worm. The displacement is recorded and quantified after each tap, and decreases over time as the worm habituates to the tapping.

pathways (Bozorgmehr et al., 2013; Timbers et al., 2013). Short-term habituation occurs after massed training with multiple taps. The observed decrement in response typically returns to baseline after several minutes. Long-term habituation results from spaced training, using several blocks of training that are interspersed by resting periods. Long-term habituation still causes a decreased response to tap 24 hours after the last training block. Additionally, even though habituation was originally classified as non-associative learning, training in the presence of certain cues increases the habituation retention (Bozorgmehr et al., 2013; Timbers et al., 2013).

1.6.7.2 Associative learning

In associative learning, worms are trained to associate specific environmental circumstances (conditioned stimulus) with advantageous or aversive conditions (unconditioned stimulus). Conditioned stimuli can vary from odors to taste or temperature changes, and are mostly associated with the presence or absence of food (unconditioned stimulus). Various associative learning paradigms have been optimized for *C. elegans*, including gustatory and olfactory learning assays.

For example, in the short-term gustatory plasticity assay (Figure 1.5) salt is used as a conditioned stimulus and paired with the absence of food, resulting in a negative association. First, a synchronized population of worms is exposed to salt in the absence of food. As learned responses have to be compared to the responses of untrained (naive) animals, another population of worms should be exposed to the unconditioned stimulus in the absence of salt. Next, worms are placed on a chemotaxis plate and allowed to choose between salt-rich and salt-poor environments. Finally, the number of worms on each area is counted to determine a chemotaxis index (Beets et al., 2012; Hukema et al., 2006).

Besides taste-based learning assays, odors can be used as conditioned stimuli. For example, the positive butanone associative memory assay (Figure 1.6) uses butanone, which is paired with the presence of food. As a result of this positive association, attraction towards butanone is enhanced. This assay allows both the study of short-term and long-term memory, as both massed and spaced training can be performed before the chemotaxis assay (Kauffman et al., 2011; Lim et al., 2016).

1.6.8 Microfluidics for the study of behavior and neural responses

While *C. elegans*' small size has many advantages, it also makes it hard to deliver local stimuli and simultaneously measure neuronal activity. Microfluidic devices allow individual *C. elegans* worms to be trapped and monitored in a microscale chip. The design of these chips can vary from simple to complex, but all require a controlled fluid flow (Albrecht and Bargmann, 2011; Chronis et al., 2007; Stirman et al., 2010). For the simpler designs, fluid flow can be provided via syringes. More complex designs may require computer-controlled

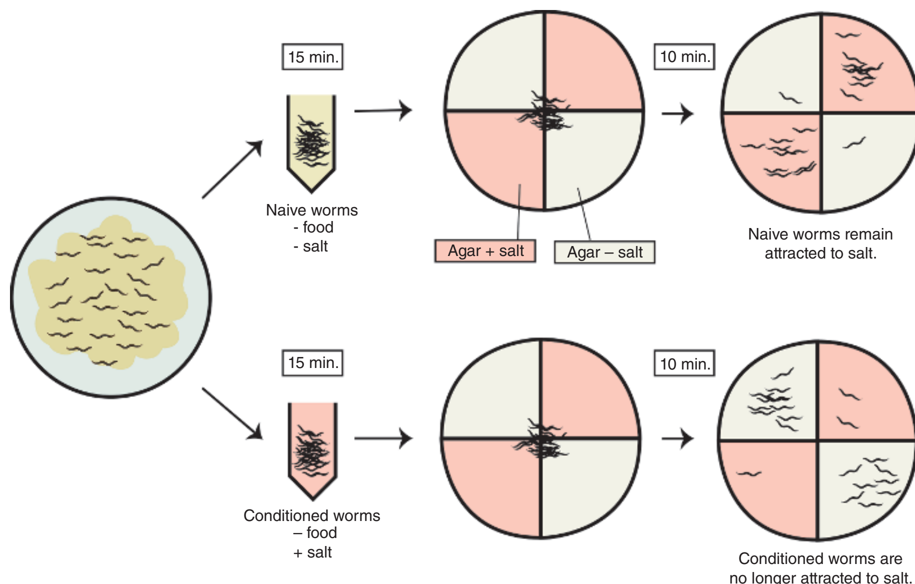

Figure 1.5 Short-term gustatory plasticity assay for associative learning of a gustatory cue, salt, and a negative stimulus, the absence of food. By default, *C. elegans* is attracted to low salt concentrations (<200 nM NaCl), because it likely serves as a proxy for food. The salt chemotaxis behavior of *C. elegans* can be quantified by placing worms on quadrant petri dishes filled with agar with or without salt. The number of worms present in quadrants with or without salt is used to calculate the chemotaxis index (= $\#_{\text{worms in salt quadrants}}$ - $\#_{\text{worms in control quadrants}}$) / ($\#_{\text{total worms scored}}$) as a measure for the worm's attraction to high salt concentrations. Naive worms, incubated in the absence of food and salt, remain attracted to salt. Conditioned worms, incubated in a salt-buffer without food, learn to associate salt with the lack of food.

valves and pumps (Lagoy and Albrecht, 2015). This relative complexity and the requirement of some general engineering knowledge is often a hurdle that needs to be overcome. As such, the technique has not yet become widely established, despite the first *C. elegans* microfluidics arrays being published nearly a decade ago (Chronis et al., 2007). While optimization may take a while, a working microfluidics platform is easy to use and allows for highly controlled and reproducible experiments. This, in combination with detailed published protocols for users unfamiliar with micro-engineering (Lagoy and Albrecht, 2015), has caused an increase in popularity for *C. elegans* microfluidics in recent years.

Microfluidics chips can be used for the detailed and controlled study of locomotion (Albrecht and Bargmann, 2011; Chronis et al., 2007) when studies on agar plates do not suffice. In addition, they have been used for the high throughput quantification of gene expression (Entchev et al., 2015). Microfluidic tools also allow studying behavioral and neural responses to tightly controlled stimuli. Such precise control of stimulus delivery can provide detailed

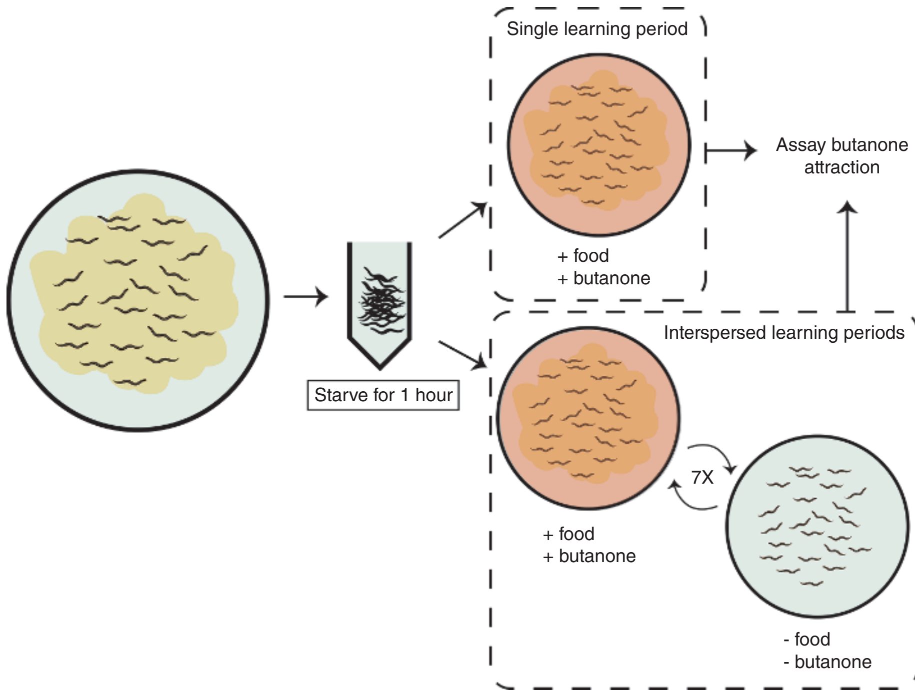

Figure 1.6 Positive butanone associative memory assay for association of an olfactory cue, butanone, with the presence of bacterial food. Well-fed young-adult *C. elegans* are collected and starved for 1 hour prior to the assay. Two learning paradigms are shown: one in which worms undergo a single learning period (massed training) in the presence of food and butanone, and second paradigm in which learning periods are alternated with short periods of starvation in the absence of butanone (spaced training). Worms undergoing massed training show an increased attraction to butanone after conditioning that lasts under 2 hours, while spaced training allows recollection of the positive butanone/food association for up to 16 hours. This figure was designed based on an experimental procedure by Kauffman et al. (Kauffman et al., 2011).

insights into the dynamics of neural responses, such as the bi-phasic response of the nociceptive ASH neurons (Wang et al., 2015).

Microfluidics is often used in combination with optogenetics or calcium imaging (Chronis et al., 2007; Lagoy and Albrecht, 2015; Stirman et al., 2010). In one of its most popular applications, i.e. the 'olfactory chip' (Chronis et al., 2007), controlled pressure is used to immobilize individual worms. The end of the trap is designed to conform to *C. elegans*' head so that the head – and thus also the worm's primary chemosensory organs, the amphids – protrudes into a different channel. A flowing stream through this channel is controlled by different valves that allow for near-instant exposure to a delivered chemical stimulus to the trapped worm's head. This can be combined with calcium imaging (see 1.7.2 and Figure 1.7) to accurately visualize neuronal responses to the provided stimulus.

1.6.9 Common pitfalls in behavioral assays

Even though behavioral assays seem straightforward, they must be performed with great care. Small changes in assay characteristics, culturing conditions and assay conditions can greatly affect the assay's outcome. Therefore, means to avoid sample variation and ensure consistency should be considered (Hart, 2006).

Major points to consider when performing behavioral assays are the specific cultivation conditions. The compounds used to make culture and assay plates should be consistent as well as the used *E. coli* strain, the thickness of assay

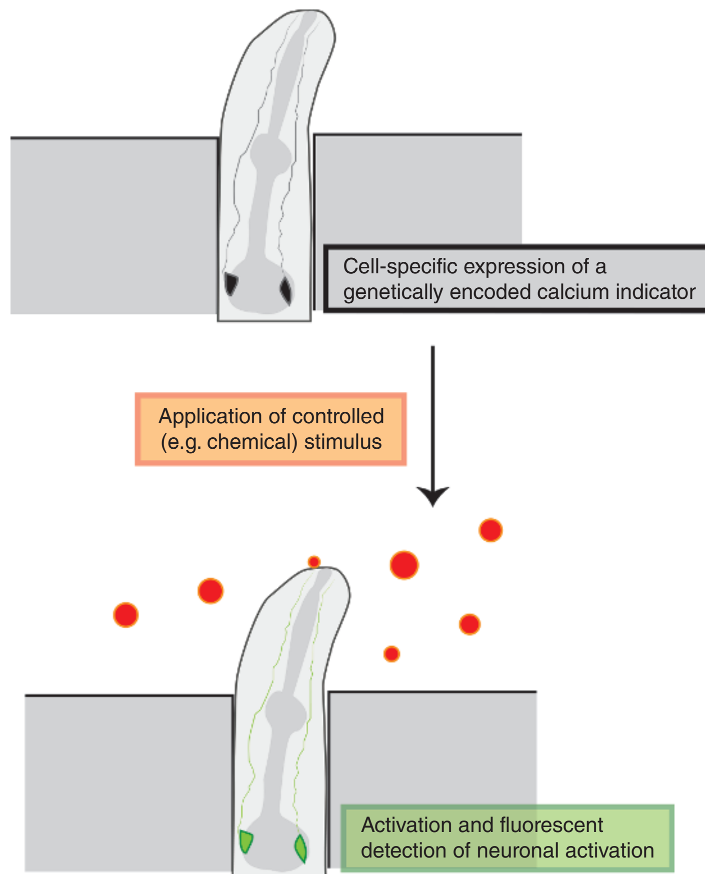

Figure 1.7 A common experimental setup combining calcium imaging and microfluidics to study the neuronal response to a controlled stimulus. The animal is restrained in a microfluidics chip allowing for stable imaging without anesthetics. A fusion protein containing a calcium responsive domain (e.g. calmodulin) and a photoactive domain (e.g. GFP) is expressed cell-specifically in the neurons of interest, marked in black. After the application of a controlled (e.g. chemical) stimulus, activation of these neurons can be observed *in vivo* by a change in fluorescent signal of the calcium indicator, marked in green.

plates, and the quantity of seeded bacteria. Additionally, freshly poured and seeded plates should be used for each assay, as plates tend to dry out, which can affect behavioral responses. It is also paramount to avoid growing worms on contaminated or food-deprived plates, as this can change their behavioral responses. Another critical point is the population's crowding and feeding status. Worms on crowded plates display different behaviors to those grown on plates with a lower number of worms. Furthermore, no starved worms should be used for behavioral assays as this influences their behavior. Performing behavioral assays on plates with or without food should also be taken into consideration, as this significantly influences the assay's outcome. Additionally, males and hermaphrodites can show distinct behaviors. Using a synchronized worm population is important when behavior differs from stage to stage or depends on age. In general, day 1 adults are used for behavioral assays, as they can be easily staged. Some experimental purposes might require worms at different stages; hence care should be given to staging the worms properly (Hart, 2006).

Ambient conditions might also affect your assay. Factors such as temperature, vibration, humidity, drafts and odors can affect an assay's outcome. For instance, when performing learning and memory assays, humidity and temperature should be tightly controlled. During locomotion or mechanosensation assays, external vibration should be avoided. Additionally, some buffers should be prepared fresh before each assay (Hart, 2006).

1.7 Neuronal circuits and the cellular basis of neuroendocrine signaling

C. elegans holds other advantages beyond the functional study of behaviors. The worm's nervous system's connectivity has been fully mapped, allowing a thorough study of the neural circuits underlying behaviors.

1.7.1 Genetic tools for unraveling neuroendocrine circuits

C. elegans' transparency offers the possibility of using fluorescent tags to study the *in vivo* expression pattern of proteins of interest. Fluorescent tags, most commonly GFP or mKate, can be directly fused to the protein in question. Alternatively, trans-splice sites allow separate expression of the target gene and fluorescent tag from a single promoter in the same transgene (Tursun et al., 2009). Functions have been described for many of *C. elegans'* neurons. As such, cellular localization of a protein can often be linked to a certain function. By transgenically restoring the expression of a protein in a mutant background, the protein's function in a biological process can be verified. Such rescue experiments can be further refined using cell-specific promoters to drive gene expression. For example, cell-specific expression of *nlp-7* and *flp-11* were recently used

to confirm that both peptide genes are required in a single secretory uterine cell to regulate egg-laying behavior (Banerjee et al., 2017).

1.7.2 Imaging *in vivo* activity of *C. elegans*' neurons

Calcium imaging (Figure 1.7) enables the study of neuronal activity in real time and *in vivo* and is most often used to measure neuronal responses in *C. elegans*. Calcium entry through voltage-gated channels plays a prime role in neuronal excitability, as *C. elegans* lacks voltage-gated sodium channels. Although chemical dye-based solutions are available, calcium imaging in *C. elegans* usually relies on genetically-encoded calcium indicators, such as GCaMP or FRET-based chameleon sensors (Kerr, 2006). Neuron depolarization causes an increase in intracellular calcium levels, which typically results in an increased fluorescent signal from the calcium indicator. This signal is easily visualized and localized to a specific neuron in the transparent *C. elegans*.

Using calcium imaging, neuronal responses to specific stimuli can be analyzed, both spatially and temporally, in immobilized or freely moving worms (Cho et al., 2017). For example, calcium imaging was used to implicate the *C. elegans* ASH neuron in adaptation to repellents in a low-food environment. This neuronal response is mediated by the neuropeptide receptor NPR-1 (Ezcurra et al., 2016). On a larger scale, this technique has been used to monitor calcium transients in an entire *C. elegans* 'brain' (i.e. up to 77 head neurons) at cellular resolution (Nguyen et al., 2015).

1.7.3 Optogenetics: targeted activation and inactivation of neurons

C. elegans was the first multicellular organism in which optogenetic manipulation of its behavior by expression of transgenic channelrhodopsins was achieved. Optogenetics relies on the ability of microbial opsins, such as channelrhodopsin, to respond to a light stimulus. Neurons expressing these proteins can be excited or inhibited by applying a flash of light of a specific wavelength. This technology provides a non-invasive tool for studying how neurons relate functionally to one another and how neuronal circuits control behavior. In a landmark experiment, channelrhodopsin-2 was expressed in the mechanosensory neurons of *C. elegans*. When exposing the animal to a flash of blue light, channelrhodopsin undergoes a conformational change resulting in the opening of a cation channel. Subsequent depolarization of the mechanosensory neuron causes the worm to reverse its movement (Boyden et al., 2005). Recently, a neuroendocrine motor neuron, RID, was identified with the help of both calcium imaging and optogenetics (Lim et al., 2016). Stimulating RID through optogenetics prolongs forward movement, showing that RID sustains the forward motor state by RFamide neuropeptide signaling. In addition, many researchers are finding ways to adapt optogenetics for new applications. Recently, a generator of reactive oxygen species was placed under

optogenetic control in *C. elegans*, allowing for rapid and precise mutagenesis (Noma and Jin, 2015).

1.7.4 Optogenetics and calcium imaging: a common pitfall

Both calcium imaging and optogenetics rely on transgenesis and fluorescence microscopy, which are well established in *C. elegans*. A challenge common to both techniques, however, is the requirement for cell-specific promoters. Calcium imaging and optogenetics have the capacity to be cell-specific, but only if their fluorescent readout (provided by genetically-encoded calcium indicators or photoactive proteins such as channelrhodopsin, respectively) can be controlled by a cell-specific promoter sequence. While many neuron-specific promoters exist in *C. elegans,* not all neurons are covered. Alternatively, cell-specific transgene expression can be achieved using combinations of promoters in Cre-LoxP or Flp-Frt systems, but these techniques are more laborious (Hubbard, 2014).

1.8 Translational considerations

The translational potential of *C. elegans* is both technical and fundamental in nature. Many techniques that are now commonly used in other model organisms, such as RNAi and GFP-based reporters, were first described and optimized in *C. elegans*. Techniques that belong to the state-of-the-art in *C. elegans* research (e.g. calcium imaging, optogenetics, connectome mapping) will likely become further optimized in this model system and more widely applied in other species in the future. Because of its ease of use, *C. elegans* provides a hotbed for tool-development in an *in vivo* context and can be considered as a first multicellular step towards widespread applications in other organisms, including humans.

Physiologically, *C. elegans* research reveals another level of translational relevance. Although *C. elegans* diverged evolutionarily from modern-day humans approximately 500-600 million years ago, many physiological and disease-related processes remain conserved. This is illustrated by OrthoList, a compendium of over 7,000 *C. elegans* genes with human orthologs (Shaye and Greenwald, 2011). Many of these genes belong to conserved neuroendocrine systems, such as the TRH, GnRH, cholecystokinin, vasopressin/oxytocin and insulin signaling systems. Models for studying neurodegenerative, metabolic and muscle-associated disorders have been generated in *C. elegans*. These include models for Alzheimer's disease in which human β-amyloid peptide is expressed in *C. elegans* muscle cells (Link, 1995), and models for Huntington's disease that express different lengths of polyglutamine stretches in muscle (Morley et al., 2002) or neuronal cells (Brignull et al., 2006). The advantages of *C. elegans* as a genetic model organism allow study of the cell biology of these disorders and have aided elucidation of molecular pathways and modifier genes

involved in specific diseases (Markaki and Tavernarakis, 2010). For example, an RNAi screen using a fluorescently tagged version of human α-synuclein recently revealed new regulators of α-synuclein aggregation (Jadiya et al., 2016). In biological and biomedical research, *C. elegans* can therefore feature both as a technological and physiological springboard for the development of methods and concepts that can have the potential to translate across model organisms.

1.9 Perspectives

C. elegans' main benefits as a model for addressing questions in neuroendocrinology are its genetic tractability, its short generation time, its fully sequenced and annotated genome and the availability of extensive tools for studying biological processes at the level of genes, neural circuits and behaviors. Reverse genetics is facilitated by established techniques for knocking out or modifying genes *in vivo*. Furthermore, behavioral readouts are straightforward, and cell-specific rescues and neural imaging or manipulation allow reconstructing the neuronal circuits underlying neuroendocrine signaling pathways.

Despite its considerable evolutionary distance from mammals and primates, many neuroendocrine pathways are well conserved in *C. elegans*, both molecularly and functionally. Recent discoveries of signaling systems that were previously thought to be poorly conserved in *C. elegans* – such as vasopressin/oxytocin- and TRH-like neuropeptide pathways - illustrate the strength of this nematode model. In sum, *C. elegans* is a first-line model for the study of the evolutionary and genetic basis of the neuroendocrine control of behavior and physiology.

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