Egg-laying*

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Abstract

***C. elegans* hermaphrodites are self-fertile, and their rate and temporal pattern of egg-laying are modulated by diverse environmental cues. Egg-laying behavior has served as an important phenotypic assay for the genetic dissection of neuronal signal transduction mechanisms. This chapter reviews our current understanding of the neuronal and neurochemical mechanisms underlying the control of egg-laying in *C. elegans*. The roles of specific neurons in the egg-laying motor circuit, which release multiple neurotramsmitters affecting distinct parameters of egg-laying muscle activity, and the possible mechanisms for sensory control of egg-laying behavior, are discussed.**

Among the motor actions of which a nematode is capable, laying an egg is among the simplest. As a consequence, egg-laying is among the first *C. elegans* behaviors to be subjected to extensive genetic analysis, and it has served as an effective behavioral assay for a number of fundamental processes in neuronal cell biology and signal transduction (e.g., trimeric G-protein signaling; Brundage et al., 1996; Koelle and Horvitz, 1996; Mendel et al., 1995; Segalat et al., 1995). This chapter focuses on our current understanding of the surprisingly intricate neural and neurochemical mechanisms that underlie egg-laying behavior and its regulation.

1. A description of egg-laying behavior

C. elegans hermaphrodites are self-fertile, producing first sperm, which are stored in the spematheca, and then oocytes. Within the first day of the L4/adult molt, hermaphrodites accumulate fertilized eggs in the uterus; a young

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adult hermaphrodite will generally have a store of 10–15 eggs in its uterus at any given time. Egg-laying occurs when specialized sex-specific muscles contract, opening the vulva and allowing eggs to be expelled. Egg-laying behavior can thus be reduced to the question of how the animal controls the timing and spatial positioning of these egg-laying events is regulated by environmental and/or homeostatic cues.

1.1. Temporal pattern of egg-laying

Under conditions favorable to egg-laying, egg-laying events (i.e. openings of the vulva leading to the expulsion of one or sometimes two eggs) occur in a specific temporal pattern. Specifically, egg-laying mostly occurs in short bursts lasting approximately 1–2 minutes, which are separated by longer quiescent periods averaging about 20 minutes in duration (Waggoner et al., 1998). Both the duration of the inactive periods between bursts and the intervals between events within a burst model as independent, exponentially-distributed random variables with different characteristic rate constants (Zhou et al., 1998). Thus, the timing of egg-laying events can be modeled as a stochastic process in which animals fluctuate between distinct behavioral states--an active phase, during which egg-laying events are frequent, and an inactive phase, during which eggs are retained. This pattern implies that the likelihood of an egg-laying event is not strongly dependent on the precise number of eggs in the uterus. If it were, the duration of an interval following the end of an active phase (during which the number of eggs in the uterus can be reduced by as many as 10) would tend to be unusually long, while the intervals following an inactive phase (during which the number of eggs in the uterus has increased) would tend to be unusually short. Since the duration of an interval is appears independent of the previous interval, it is unlikely that a mechanism exists to maintain a precise number of eggs in the uterus. More generally, the fact that the egg-laying pattern can be broken down into these specific temporal parameters may imply specific roles for particular neurons or neurotransmitters in egg-laying modulation.

1.2. Modulation by sensory cues

Egg-laying is regulated by a number of environmental conditions. For example, mechanical stimulation such as vibration of the culture medium inhibits egg-laying, an effect that requires the body touch receptors ALM and PLM (Sawin, 1996). Hypertonic salt solutions such as M9 salts also strongly inhibit egg-laying (Horvitz et al., 1982). Finally, the egg-laying rate in the presence of abundant food is significantly higher than in the absence of food (Trent, 1982). This effect has been demonstrated using several experimental paradigms, and may in fact be mediated by multiple regulatory mechanisms. The modulation of egg-laying rate by food abundance appears to require the neuropeptides encoded by the *flp-1* gene, since *flp-1* deletion mutants show a food-modulation defect (Waggoner et al., 1998) which is rescued by a *flp-1* wild-type transgene (L. Waggoner, unpublished). Another molecule that appears to be involved in the control of egg-laying by food is the cyclic GMP-dependent protein kinase EGL-4 (L'Etoile et al., 2002; Fujiwara et al., 2002). Mutations in *egl-4* slow the rate of egg-laying on food and render animals insensitive to food modulation of egg-laying rate (Daniels et al., 2000). This phenotype is suppressed by mutations in *daf-3* and *daf-5* (Trent et al., 1983), which encode proteins involved in TGF-β signaling (da Graca et al., 2004; Patterson et al., 1997). Mutations in other genes encoding TGF-β pathway members, such as *daf-1*, *daf-4*, *daf-7*, *daf-8* and *daf-14* (reviewed in (Patterson and Padgett, 2000), also show egg-laying defective (as well as dauer-constitutive) phenotypes that are suppressed by *daf-3* and *daf-5* (Thomas et al., 1993; Trent et al., 1983). Thus, a pathway involving PKG and TGF-β signaling appears to play a critical role in the sensory control of egg-laying. At least some of these genes (e.g., *daf-7* (Schackwitz et al., 1996), *egl-4* (L'Etoile et al., 2002) are known to function in chemosensory neurons, suggesting that they may function in or downstream of sensory neurons that influence the egg-laying circuitry. However, the identities of these sensory neurons, and neural mechanisms by which they might influence egg-laying behavior, are not known.

1.3. Coordination with other behaviors

In addition to receiving modulatory input from sensory cells, egg-laying is also coordinated with other motor programs. Specifically, egg-laying is temporally correlated with locomotion. Immediately prior to an egg-laying event, there is a transient increase in locomotor velocity; in addition, reversals are inhibited during egg-laying (Hardaker et al., 2001). It is interesting to note that in *Prionchulus punctatus*, a free living predatory species, egg-laying and locomotion are correlated in the converse manner: egg-laying occurs only during periods of inactivity (Maertens, 1975). The coordination of egg-laying and locomotion in *C. elegans* appears to be mediated by feedback from the HSN motorneurons to interneurons in the head that promote forward movement. The direct post-synaptic output of HSN is to AVF, which in turn synapses onto the forward command interneuron AVB. Ablation of the HSNs or the AVFs or interference with serotonin neurotransmission leads to the absence of the

velocity burst prior to egg-laying events (Hardaker et al., 2001). These results imply that the activity of the HSNs during periods of active egg-laying also modulates interneurons controlling locomotion. This observation raises the possibility that the behavioral states observed for egg-laying may in fact represent general states of neural circuit function that could affect diverse, seemingly unrelated motor outputs.

2. Functional roles of egg-laying circuitry components

The structure of the egg-laying circuit, defined in the anatomical studies of White and colleagues (White et al., 1986), is relatively simple. Egg-laying occurs through a simple motor program involving specialzed smooth muscle cells, whose contraction opens the vulva and compresses the uterus so that eggs can be deposited into the environment. The egg-laying muscles receive synaptic input from two classes of motorneurons, the 2 HSNs and the 6 VCs. The specific functions of these cells are discussed below.

2.1. Egg-laying muscles

A total of 16 muscle cells are likely to be involved in the mechanics of egg-laying. Of these, the 4 vm2 vulval muscles have been shown to be particularly critical. The vm2s are the only egg-laying muscles receiving significant synaptic input from neurons (White et al., 1986). They are arranged in an cross shape, with their apical ends attached to the vulva, and are electrically-coupled to one another through gap junctions. Ablation of the vm2s completely abolishes egg-laying, indicating that contraction of the vm2s is essential for opening the vulva (M. Stern, personal communication). While vm2 vulval muscle cells can contract individually, egg-laying events appear to involve simultaneous contraction of several if not all the vulval muscles.

Two additional classes of hermaphrodite-specific smooth muscles are thought to be involved in egg-laying. The 4 vm1 vulval muscles are arranged in an X-shaped pattern similar to the vm2s, and are also thought to be involved in opening the vulva. The vm1s are electrically coupled to the vm2s (White et al., 1986), but receive no significant synaptic input and do not markedly alter egg-laying when ablated (M. Stern, personal communication). There are also 8 uterine muscles, which form bands surrounding the anterior and posterior arms of the uterus. Contraction of these muscles might be expected to promote egg-laying by constricting the uterus and thus pushing eggs out of the vulva. However, ablation of all 8 uterine muscles does not cause a gross egg-laying defect (M. Stern, personal communication).

2.2. HSN motorneurons

The HSNs are hermaphrodite-specific motorneurons (in the male they undergo programmed cell death) that play a central role in egg-laying behavior. The HSN cell bodies are located lateral and slightly posterior to the vulva, and extend a long process ventrally into the ventral nerve cord and then anteriorly into the nerve ring. In the vulval area, the HSNs make extensive neuromuscular junctions with the vm2 vulval muscles, and also direct synaptic output to the VC5 motorneurons. The HSNs also receive synaptic input near the cell body from the PLM mechanosensory neurons (White et al., 1986). Perhaps surprisingly for a motorneuron, in the nerve cord the HSNs are primarily presynaptic. The HSNs use at least three neurotransmitters: serotonin (Horvitz et al., 1982; Desai et al., 1988), acetylcholine (Duerr et al., 2001), and one or more neuropeptides (Schinkmann and Li, 1992; Kim and Li, 2004).

The HSNs are critical to the normal execution and regulation of egg-laying. Ablation of the HSNs results in a strong reduction in egg-laying rate, and many egg-laying defective mutants have abnormal HSN development or function (Trent et al., 1983; Desai and Horvitz, 1989). Particularly useful for genetic studies are semidominant *egl-1* mutations, which activate the male-specific cell death pathway and thus lead specifically to the loss of HSNs in hermaphrodites (Conradt and Horvitz, 1998). Analysis of the timing of egg-laying events in HSN-ablated animals indicates that the HSNs are specifically important for inducing the onset of egg-laying active phases; clusters of egg-laying events are about 3-fold less frequent than in normal animals, though egg-laying within these bursts occurs normally (Waggoner et al., 1998). Since exogenous serotonin rescues these egg-laying defects (Trent et al., 1983), it is likely that the HSNs promote egg-laying at least in part by releasing serotonin as a neuromodulator. Calcium imaging studies have shown that serotonin acts directly on the vulval muscles to increase the frequency of spontaneous calcium transients (Shyn et al., 2003), an effect most likely mediated by the Gq homologe EGL-30 (Bastiani et al., 2003). However, since serotonin-deficient mutants (e.g. *tph-1*) have less severe egg-laying defects than HSN-ablated animals (Weinshenker et al., 1995; Kim et al., 2001), the HSNs may also release another neuromodulator (perhaps a peptide) that functions semi-redundantly with serotonin to promote egg-laying. Interestingly, serotonin appears to have a converse effect on the HSNs themselves; spontaneous neural activity in the

HSNs is silenced by exogenous serotonin, an effect requiring the Go homologue GOA-1 (Shyn et al., 2003). This effect may account at least in part for the inhibition of egg-laying caused by chronic exposure to high concentrations of serotonin (Schafer et al., 1996).

Since the HSNs are the major synaptic link between the egg-laying circuit and the rest of the nervous system, it is likely to function as an important conduit for regulatory feedback to and from the egg-laying motor program. As noted above, the HSNs appear to be critical for coordinating egg-laying and locomotion. Surprisingly, the evidence that HSN is involved in communication between head neurons and the egg-laying circuit is less clear. For example, animals lacking the HSNs are still capable of modulating their egg-laying rate in response to food abundance, suggesting that humoral or other extrasynaptic modulation of the vulval muscles and/or VCs may mediate this regulation (Waggoner et al., 2000b). Exactly how the activity of the HSNs is controlled, and how this regulation relates to egg-laying behavior is one of the key unanswered questions about *C. elegans* egg-laying.

2.3. VC motorneurons

The VCs are 6 hermaphrodite-specific neurons in the ventral nerve cord; their male homologues are the serotonergic CP motorneurons involved in tail curling. The VCs can be further subdivided into two classes: the vulva-proximal VC4 and VC5 neurons, and vulva-distal VC1-3 and VC6. VC4 and VC 5 direct their synaptic output exclusively to the vulval muscles and to the other VCs. These neurons are chlolinergic (Duerr et al., 2001) and express one or more RF-amide neuropeptides (Schinkmann and Li, 1992). In addition, they express the vesicular monoamine transporter (Duerr et al., 1999) and stain weakly for serotonin (Duerr et al., 2001), though they are not reported to express the serotonin biosynthetic enzyme tryptophan hydroxylase (Sze et al., 2000). Thus, these neurons probably also release a monoamine, possibly serotonin, which might enter the cells by reuptake from vulval muscle neuromuscular junctions. VC5 in particular receives significant synaptic input from the HSNs. The other VCs (VC1-3 and VC6) make fewer neuromuscular junctions with the vulval muscles than VC4 and VC5. Additionally, unlike the proximal VCs, which extend only short processes that innervate the vulval muscles, the distal VCs also extend a longer process into the ventral nerve cord that directs synaptic output to both the body muscles and the D-class GABAergic motorneurons. These neurons also do not express CAT-1/VMAT, and are therefore not thought to be aminergic (Duerr et al., 2001).

The effects of the VC neurons on egg-laying behavior, as assessed by neuronal ablation, are less striking than those seen for the HSNs. However, several lines of evidence suggest that at least the vulva-proximal VCs (VC4 and VC5) stimulate vulval muscle activity. Ablation of VC4 and VC5 in an *egl-1* mutant background significantly enhances the egg-laying defective phenotype caused by loss of the HSNs (Waggoner et al., 1998). Moreover, *in vivo* calcium imaging studies have shown that VC activity is temporally correlated with vulval muscle movement (Shyn et al., 2003; M. Zhang and W. Schafer, unpublished). Seemingly paradoxically, there is also compelling evidence for an inhibitory role for the VCs in egg-laying. In particular, a number of egg-laying-constitutive mutants have been shown to be defective in VC morphology or neurotransmitter release (Bany et al., 2003) or have an apparent focus of action in the VCs (Schafer and Kenyon, 1995). In addition, VC4/VC5 ablated animals accumulute fewer unlaid eggs, suggesting that their overall rate of egg-laying is elevated (Bany et al., 2003).

The question of the VCs' role in egg-laying is closely related to the role of acetylcholine, a key VC neurotransmitter. Nicotinic agonists strongly stimulate egg-laying (Trent et al., 1983; Weinshenker et al., 1995), an effect requiring the function of specific nicotinic receptors in the vulval muscles (Weinshenker et al., 1995); (Waggoner et al., 2000a). In contrast, chronic increases in cholinergic neurotransmission (e.g. in cholinesterase-deficient mutants) inhibits egg-laying in a manner dependent on the HSN-expressed muscarinic receptor GAR-2 (Bany et al., 2003). As for serotonin, these inhibitory effects of acetylcholine on the HSNs may involve the Go signal transduction pathway (Moresco and Koelle, 2004). A model consistent with all existing data is that the release of acetylcholine from the VCs stimulates vulval muscle contraction through nicotinic receptors while feedback inhibiting the HSNs through muscarinic receptors . In addition, it is possible that other neurotransmitters (such as neuropeptides) expressed in the VCs may play stimulatory or inhibitory roles in egg-laying behavior.

3. Perspectives

The apparent anatomical simplicity of the *C. elegans* egg-laying circuit belies a surprising complexity at the molecular and neurochemical levels. The use of multiple neurotransmitters by the egg-laying motorneurons allows a simple circuit to generate complex temporal patterns of behavior. Among the central questions for future investigation is how sensory information modulates the rate of egg-laying. The structure of the *C. elegans* nervous system provides few clues as to how information could be conveyed from sensory neurons such as the amphid

chemoreceptors to the egg-laying motorneurons. Potentially, neurohormonal or other extrasynaptic signaling may play an important role in these processes, raising the possibility that the cellular network involved in egg-laying control is considerably more complex than previously suspected.

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