Acetylcholine*

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Abstract

Acetylcholine is the major excitatory neurotransmitter at nematode neuromuscular junctions, and more than a third of the cells in the *C. elegans* nervous system release acetylcholine. Through a combination of forward genetics, drug-resistance selections, and genomic analysis, mutants have been identified for all of the steps specifically required for cholinergic function. These include two enzymes, two transporters, and a bewildering assortment of receptors. Cholinergic transmission is involved, directly or indirectly, in many *C. elegans* behaviors, including locomotion, egg laying, feeding, and male mating.

1. Introduction to cholinergic metabolism

Acetylcholine (ACh) was the first substance proven to be a neurotransmitter (Loewi, 1921). It was identified in *Ascaris* and other nematodes in 1955 by Helen Mellanby (Mellanby, 1955), and was subsequently shown to be an excitatory transmitter at nematode neuromuscular junctions (del Castillo et al., 1963; del Castillo et al., 1967).

As shown in Figure 1, ACh is synthesized by choline acetyltransferase (ChAT), and is loaded into synaptic vesicles by the vesicular acetylcholine transporter (VAChT). The synaptic vesicle lumen is acidified by the action of an ATP-dependent proton pump located in the synaptic vesicle membrane. The pH gradient between the vesicle lumen and the cytoplasm provides the driving force for ACh transport; the VAChT essentially “exchanges” ACh for protons. The docking and priming of synaptic vesicles, and their calcium-stimulated fusion with the cell membrane are all general processes that are independent of the neurotransmitter contained in the vesicles (and are described elsewhere in this volume). Following synaptic vesicle fusion and transmitter release, the ACh diffuses within the synaptic cleft and activates acetylcholine receptors (AChRs), usually located on post-synaptic cells. For most other neurotransmitters (e.g., GABA, dopamine, serotonin), the action of the transmitter is terminated by transporter-mediated removal of the transmitter from the synaptic cleft. The action of acetylcholine, however, is terminated by direct enzymatic hydrolysis of the neurotransmitter in the synaptic cleft by acetylcholinesterase (AChE). The resulting choline is then transported back into the presynaptic neuron by a high affinity choline transporter (HAChT, or ChT); this choline is then available for the synthesis of additional ACh.

In subsequent sections, each of these steps will be described, along with properties and subcellular localization of the proteins, their expression pattern, and mutant phenotypes. I then discuss ACh-mediated behaviors, and some additional aspects of cholinergic biology.

2. Cholinergic pharmacology and drug-resistant mutants

2.1. Background

Sydney Brenner first reported the isolation of drug-resistant mutants of *C. elegans*; the drugs he tested were the cholinesterase inhibitor lannate and the acetylcholine receptor agonist tetramisole (Brenner, 1974). Since then, the use of cholinergic drugs for mutant isolation has been extremely useful. Many of the mutations identified by drug resistance turned out to be alleles of genes previously identified as Uncs (e.g., *unc-17*, *unc-29*), yet a significant number of the drug resistance mutations were in previously unidentified loci (e.g., *snt-1*, *ric-8*, *lev-1*).

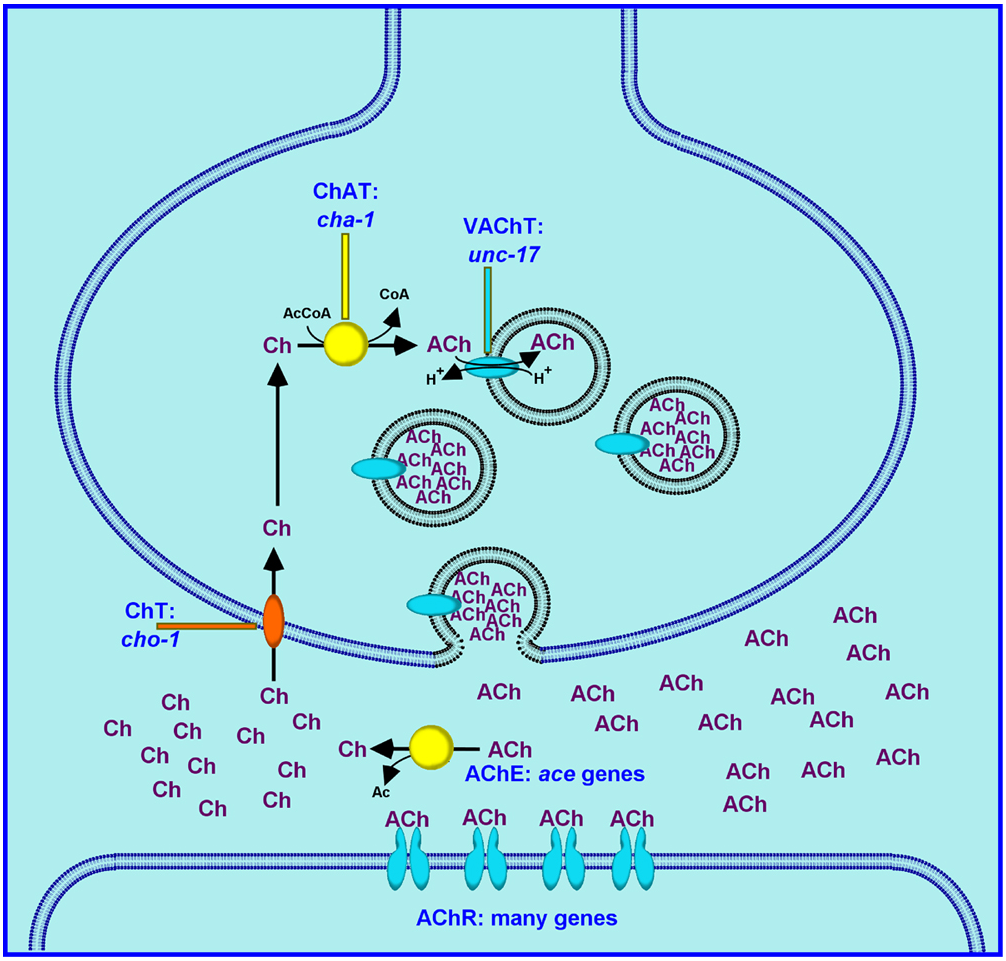

Figure 1. Cholinergic enzymes and transporters.

Mutations in a large number of genes can confer resistance, and for many of these genes, null alleles are viable; thus mutant screens and selections have been quite productive (Lewis et al., 1980b; Nguyen et al., 1995; Miller et al., 1996). Many cholinergic drugs (some of which are mentioned in subsequent sections) have been used in the analysis of specific mutants and behaviors; however, the two that have had the most significant impact on *C. elegans* neurobiology are aldicarb and levamisole.

2.2. Aldicarb and Ric mutants

Although lannate was initially used for such studies (Brenner, 1974), and trichlorfon has also been used (Hosono et al., 1989), the carbamate acetylcholinesterase inhibitor aldicarb (see Figure 2) has become the reagent of choice for evaluation of the "Ric" phenotype (for **R**esistance to **I**nhibitors of **C**holinesterase). There are more than 50 genes known which can mutate to confer a Ric phenotype. The direct consequence of inhibiting AChE is a buildup of synaptic ACh, and since ACh is the excitatory transmitter at neuromuscular junctions, the excess ACh causes a hypercontracted paralysis. In principle, there should be two major gene classes which can mutate to confer aldicarb resistance. A mutation which disrupts ACh reception or any portion of the postsynaptic response to ACh would be expected to confer resistance (the muscle wouldn't "know" or "care" that there was excess ACh on its doorstep). In addition, however, any mutation which disrupted ACh synthesis, vesicular loading or release would lead to less ACh being released by the motor neurons, so there wouldn't be as much ACh to build up in the first place. A third possibility, mutation of an acetylcholinesterase subunit, would presumably require a very specific gain-of-function mutation, and is expected to be extremely rare.

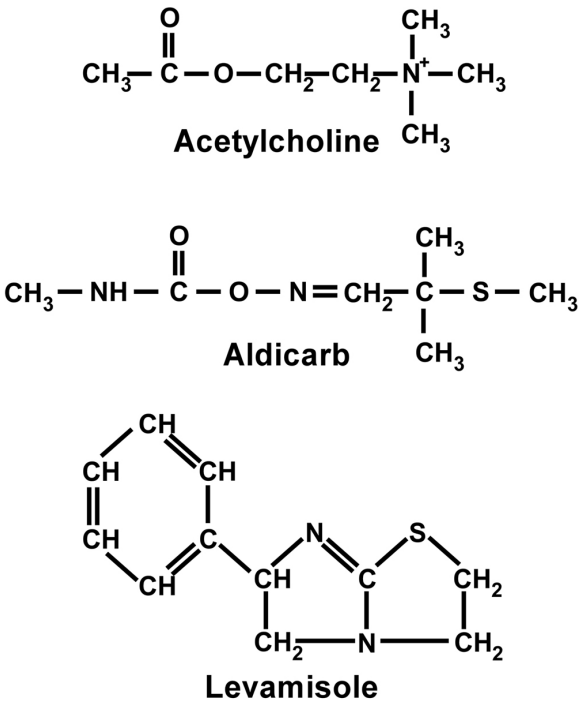

Figure 2. The structures of acetylcholine, aldicarb, and levamisole.

Operationally, there are two strategies to determine/assess resistance to aldicarb. One can measure behavioral impairment following "acute" exposure to aldicarb, for example, the percent of animals totally paralyzed after 4 hrs as a function of aldicarb concentration (Nonet et al., 1993), or the percent of animals totally paralyzed as a function of time at, e.g., 2 mM aldicarb (Schade et al., 2005). An alternative is to measure growth and development in the presence of aldicarb, i.e., "chronic" exposure (Nguyen et al., 1995; Miller et al., 1999). Most mutageneses have employed a selection scheme based on growth and reproduction in the presence of aldicarb.

Although a few of the Ric mutations clearly affect cholinergic targets (e.g., *cha-1*, *unc-17*, *ric-3*, described in subsequent sections), most of them are in genes involved in the release of all neurotransmitters. Thus, for example, the first mutations in *snt-1* (synaptotagmin), *snb-1* (synaptobrevin), and *ric-8* (synembryn) were isolated in large-scale selections for aldicarb resistance (Nonet et al., 1993; Nonet et al., 1998; Miller et al., 2000). As a result, aldicarb resistance in *C. elegans* is often equated with decreased neurotransmitter release, an unfortunate oversimplification. Nevertheless, a number of successful forward genetic screens have identified many important presynaptic components (Nguyen et al., 1995; Miller et al., 1996; Yook et al., 2001), and a recent genome-scale reverse genetic approach using aldicarb to identify genes affecting synaptic function has been described (Sieburth et al., 2005).

2.3. Aldicarb, Hic mutants, and G-protein pathways

In contrast to the Ric phenotype, mutants have been described with the opposite "Hic" phenotype (**H**ypersensitivity to **I**nhibitors of **C**holinesterase); and while many mutants with reductions in transmitter release are associated with aldicarb resistance, a number of mutants with apparent elevated release of acetylcholine are characterized by hypersensitivity to aldicarb (Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999; Miller and Rand, 2000; Reynolds et al., 2005; Schade et al., 2005).

A particularly fruitful use of aldicarb mutants has been in the elaboration of a G-protein-coupled regulatory network that regulates ACh release from the ventral cord motor neurons (see Heterotrimeric G proteins in *C. elegans*). A core pathway in this network includes RIC-8, $G\alpha_q$ (EGL-30) and phospholipase Cβ (EGL-8) and leads to production of DAG, which stimulates transmitter release; loss-of-function mutations in these components lead to decreased locomotion, decreased egg laying, and aldicarb resistance (Miller et al., 1999; Lackner et al., 1999; Miller et al., 2000; Reynolds et al., 2005). Conversely, gain-of-function mutations in this core pathway lead to the opposite phenotypes: hyperactive locomotion and egg laying, and hypersensitivity to aldicarb. Negative regulators of this

pathway include $G\alpha_o$ (GOA-1) and DAG kinase (DGK-1), which reduce DAG levels and reduce transmitter release; loss-of-function mutations in such negative regulators lead to hyperactivity and aldicarb hypersensitivity (Nurrish et al., 1999). Many of the mutants that defined this signaling network were either isolated on the basis of altered response to aldicarb, or their response to aldicarb provided critical data for the interpretation of their function(s).

2.4. Levamisole and Lev mutants

Levamisole (see Figure 2) is a potent cholinergic agonist, and is often used as a pesticide. It leads to a hypercontracted paralysis of wild-type nematodes, usually followed by relaxation and death (Lewis et al., 1980b). Levamisole has been shown to bind specifically to one of the two receptor types present in body-wall muscle, and has therefore been an extremely valuable tool in the molecular and physiological analysis of *C. elegans* AChR subunits (see Behavior section of WormMethods) . Operationally, the sensitivity or resistance of a given strain may be determined by monitoring either the time-course of paralysis or the extent of body-shortening due to muscle hypercontraction. The majority of levamisole-resistance genes were identified in several large-scale mutageneses by Jim Lewis (Lewis et al., 1980b; Lewis et al., 1980a). Mutations in several genes conferred strong resistance to levamisole, and also a mild uncoordinated phenotype: the AChR subunit-encoding genes *unc-29*, *unc-38*, and *unc-63*, and the accessory protein encoding genes *unc-50* and *unc-74* (Lewis et al., 1980b). There were also mutations conferring mild resistance to levamisole and normal locomotion; these identified the receptor subunit-encoding genes *lev-1* and *lev-8*, and the accessory protein encoding gene *lev-10*. These genes are all described below in Sections 6 and 8.

3. Acetylcholine synthesis and vesicular loading

3.1. The ChAT (CHA-1) protein

Choline acetyltransferase catalyzes the acetylation of choline by acetyl-Coenzyme A (see Figure 1). The *C. elegans* enzyme has been partially purified and characterized (Rand and Russell, 1985); it is similar to the vertebrate and *Drosophila* ChAT proteins. Immunolocalization studies indicate that most of the ChAT protein is synaptic, but some immunoreactivity appears to be generally cytoplasmic (J. Duerr and J. Rand, unpublished). The synaptic protein is associated with a vesicular compartment, most likely synaptic vesicles, because it was mislocalized in *unc-104* mutants. Both the synaptic and the cytoplasmic staining were decreased in particular *cha-1* mutant strains and increased in transgenic lines which overexpress ChAT (J. Duerr and J. Rand, unpublished).

3.2. The VAChT (UNC-17) protein

The vesicular acetylcholine transporter was first cloned and characterized in *C. elegans* (Alfonso et al., 1993), where it was shown to be the product of the *unc-17* gene. Structurally, the UNC-17 (VAChT) protein has 12 transmembrane domains, with cytoplasmic (i.e., extravesicular) N- and C-termini, and is closely related to the vesicular monoamine transporters (VMATs). Immunoreactivity with anti-UNC-17 antibody preparations in wild-type animals is punctate and colocalizes with the synaptic vesicle protein synaptotagmin, suggesting that UNC-17 is associated with synaptic vesicles (Alfonso et al., 1993). This punctate staining is mislocalized in *unc-104* mutants, providing additional support to the conclusion that UNC-17 is an integral membrane protein of synaptic vesicles.

3.3. Expression of ChAT and VAChT

Although there are several formal criteria used by neurobiologists to determine if a neuron is cholinergic, the unofficial criterion is expression of ChAT. Operationally, this means either immunolocalization of ChAT or expression of a cholinergic reporter (discussed below). By such criteria, approximately 120 neurons in the *C. elegans* hermaphrodite are cholinergic. The majority of these cells are motor neurons. These include the AS, DA, DB, VA, VB, and VC motor neuron classes of the ventral nerve cord, the sublateral motor neuron classes SAA, SAB, SIA, SIB, SMB, and SMD (Rand and Nonet, 1997), and the HSN cells (Duerr et al., 2001).

3.4. The cholinergic locus

Genetic and molecular studies showed that *cha-1* and *unc-17* were part of a complex gene locus and transcription unit, with the *unc-17* gene nested in the long first intron of *cha-1* (see Figure 3; Rand, 1989; Alfonso et al., 1994a). Thus, the sequential steps of acetylcholine synthesis and vesicle-loading are encoded by different genes within a single, complex transcription unit. Subsequent studies demonstrated that a similar nested structure of these

genes is present in mammals and insects (Erickson et al., 1994; Kitamoto et al., 1998). This is now commonly called the cholinergic gene locus, or CGL, and is present in all metazoans thus far examined (Eiden, 1998). It is noteworthy that the genes encoding enzymes and transporters for other neurotransmitters are not organized in a comparable way.

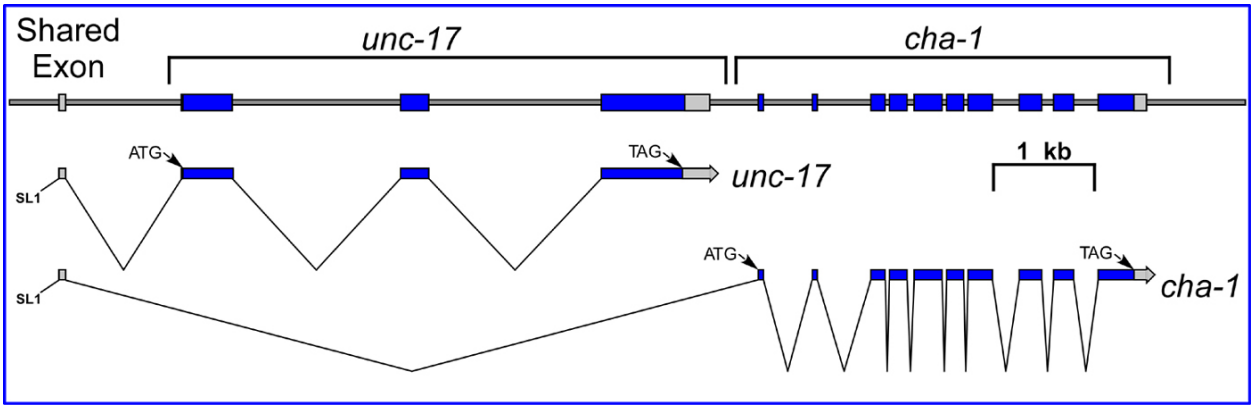

Figure 3. The *unc-17* - *cha-1* locus.

As described below, the *unc-17* promoter regulates the expression of both genes, and may therefore be considered as a "cholinergic" promoter.

3.5. *cha-1 and unc-17* mutants

unc-17 mutants were first described by Brenner (Brenner, 1974), and *cha-1* mutants were described 10 years later (Rand and Russell, 1984). More than a dozen *cha-1* alleles and more than 20 *unc-17* alleles have now been described (Brenner, 1974; Rand and Russell, 1984; Hosono et al., 1985; Rand, 1989; Alfonso et al., 1993; Alfonso et al., 1994a; Alfonso et al., 1994b; Zhao and Nonet, 2000; Zhu et al., 2001). Hypomorphic *cha-1* mutants (including animals homozygous for the reference *p1152* allele) are coily, uncoordinated, and jerky in reverse; they are also small, slow-growing, and resistant to aldicarb (Rand and Russell, 1984). Null alleles of *cha-1* are lethal - the animals hatch, but they are essentially immobile, and do not grow or feed (Rand, 1989; Alfonso et al., 1993). In general, the phenotypes of hypomorphic *unc-17* mutants (including animals homozygous for the *e245* reference allele) are similar to those of *cha-1*: animals are small, slow-growing, coiling Uncs, jerky in reverse, and resistant to cholinesterase inhibitors (Brenner, 1974; Rand and Russell, 1984). Null *unc-17* mutants are lethal, with a phenotype quite similar to *cha-1* null mutants (Alfonso et al., 1993). The lethality of *unc-17* null mutants and the similarity of their phenotype to *cha-1* nulls argue that vesicular loading is an obligatory step in ACh release.

The molecular analysis of the region has helped to explain some genetic anomalies. Even though in general, *cha-1* mutations and *unc-17* mutations behaved as two discrete complementation groups, there were a few mutations that failed to complement both *cha-1* and *unc-17* alleles (Rand and Russell, 1984). These fell into two classes, termed α and β. Fine-structure genetic mapping indicated that the single α allele mapped between *cha-1* and *unc-17*, but the two β alleles mapped well to the right of both loci (Rand, 1989). Subsequent molecular analysis revealed that the α allele corresponds to a small deletion between *unc-17* and *cha-1* (Alfonso et al., 1994a), and the β mutations are in the promoter region upstream of the *unc-17* coding sequence (D. Frisby and J. Rand, unpublished). The observed noncomplementation between the β mutations and *cha-1* alleles confirms that the promoter region upstream of *unc-17* drives both *unc-17* and *cha-1* transcription (see Figure 3).

4. Choline transport

4.1. The CHO-1 choline transporter

Like the vesicular acetylcholine transporter, the plasma membrane choline transporter was first cloned and characterized in *C. elegans* (Okuda et al., 2000). The ChT protein (CHO-1) is a member of a class of sodium-dependent transporters with substrates such as glucose (SGLT1) and inositol (SMIT1). It is best modeled as a 13-transmembrane domain protein, with a short N-terminus oriented extracellularly, and an extended, C-terminus oriented into the cytoplasm (Apparsundaram et al., 2000; Wang et al., 2001). Assays performed in embryonic cell culture showed that the *C. elegans* CHO-1 transporter was sodium- and chloride-dependent, with an apparent K_m for choline of 0.66 μM, comparable to mammalian transporters (Matthies et al., 2006).

Reporter studies indicate that *cho-1* is expressed in most or all cholinergic neurons (Matthies et al., 2006). Within these neurons, a CHO-1-GFP fusion protein was localized to synaptic regions and was associated with a vesicular compartment, because its localization was dependent on UNC-104 (Matthies et al., 2006). This is consistent with results from mammalian systems demonstrating that the choline transporter is localized to apparent synaptic vesicles (Ferguson et al., 2003).

4.2. The *cho-1* gene and mutants

The reference allele, *tm373*, is a precise 1695 bp deletion which eliminates more than half of the *cho-1* coding sequence; it is almost certainly a null allele. Animals homozygous for *tm373* are viable, and their growth and development appear to be normal. However, although *cho-1* mutants have little difficulty crawling on agar, they swim somewhat more slowly that wild-type animals, and become paralyzed ("fatigued") more quickly than wild-type animals during prolonged swimming in liquid (Matthies et al., 2006). It is tempting to speculate that there is enough endogenous choline and/or low-affinity choline uptake activity (see below) in the *cho-1* mutant nerve terminals to support some ACh synthesis even without any high-affinity reuptake activity, and that this level of ACh synthesis is sufficient for locomotion in a calm environment. However, this level of ACh is not adequate to support the increased demands of vigorous swimming, and the *cho-1* motor neurons appear to become depleted of transmitter. This provides genetic-based evidence for a functional coupling of choline transport and ACh synthesis, and supports previous models based on other methods (Jope and Jenden, 1980; Collier, 1988; Bussiere et al., 2001).

4.3. Sources of choline

If choline is indeed rate-limiting for acetylcholine synthesis under some circumstances, what determines the availability of choline? In *C. elegans*, choline is synthesized in the form of phosphocholine through progressive N-methylation of phosphoethanolamine by the PMT-1 and PMT-2 methyltransferases (Palavalli et al., 2006). Choline may also be produced through cleavage of phosphatidylcholine by phospholipase D (PLD-1). Choline is thus a product of lipid metabolism and lipid turnover. The activities of these enzymes have not been analyzed in cholinergic neurons.

4.4. Other choline transporters

SNF-6 is a post-synaptic choline/acetylcholine transporter (Kim et al., 2004). It is a member of the family of plasma membrane transporters which includes the serotonin transporter (MOD-5), the dopamine transporter (DAT-1), and the GABA transporter (SNF-11). The only two substrates identified for SNF-6 were choline and acetylcholine; both substrates had comparable K_m values, but the V_{max} for choline was about seven times the V_{max} for acetylcholine (Kim et al., 2004). This appears to be a nematode-specific transport activity. The SNF-6 protein is expressed in muscles, and is preferentially localized to neuromuscular junctions. *snf-6* mutants are somewhat uncoordinated and mildly hypersensitive to aldicarb. This transport activity may provide a mechanism for rapid clearance of synaptic ACh and/or choline during periods of high transmitter release.

Most species (and apparently *C. elegans* as well) also express several "low-affinity" choline transport activities with broad tissue distributions. The CTL proteins in mammals (corresponding to the *chtl-1* gene product) are sodium-independent transporters of choline and other organic cations (O'Regan et al., 2000). There is also a family of at least five organic cation transport (OCT) proteins in mammals with broad, overlapping substrate specificities (including choline; Friedrich et al., 2001). *C. elegans* members of this family include OCT-1 (Wu et al., 1999), OCT-2 (listed in WormBase with no data), plus 3 or 4 others in WormPep which are mostly uncharacterized. The involvement, if any, of these transport activities in cholinergic function is unknown.

5. Acetylcholinesterases

5.1. AChE proteins

Biochemical analysis of *C. elegans* homogenates led to a complicated assortment of AChE forms with different molecular weights, subunit composition, and hydrophobicity; however, three classes of AChE activity (A, B, and C) could be distinguished on the basis of substrate affinity, inhibitor specificities, and detergent sensitivities (Johnson and Russell, 1983; Kolson and Russell, 1985). Vertebrates express two families of cholinesterase activity, the "true" acetylcholinesterases and the "pseudo" or butyrylcholinesterases. However, the three *C. elegans* AChE activity classes do not correspond in any clear way to the vertebrate enzyme classes (Johnson and Russell, 1983).

The active site-containing subunits associated with the A, B, and C classes have been shown to be the gene products of the *ace-1*, *ace-2*, and *ace-3* genes, respectively (Johnson et al., 1981; Culotti et al., 1981; Kolson and Russell, 1985; Combes et al., 2000). A fourth gene, *ace-4*, has been described, but the protein it encodes appears not to be enzymatically active (see below).

The three AChE classes were subsequently shown to include a number of forms with different association states. Class A (ACE-1) subunits self-associate, forming amphiphilic tetramers; these tetramers may also bind to a hydrophobic noncatalytic subunit which is poorly characterized (Combes et al., 2000). Class B (ACE-2) and Class C (ACE-3) subunits form glycolipid-anchored homodimers (Combes et al., 2000). The Class C AChE activity represents only a few percent of the total *C. elegans* AChE activity (Kolson and Russell, 1985), yet it is noteworthy in several ways. Its apparent K_m of 16-18 nM is approximately 3 to 4 orders of magnitude lower than K_m values for Classes A and B, as well as vertebrate AChEs (Johnson and Russell, 1983; Kolson and Russell, 1985). The Class C activity is also quite resistant to cholinesterase inhibitors (Kolson and Russell, 1985). Class C-like AChE activity with these properties has been identified in other nematode species, but is not present in vertebrates (Kolson and Russell, 1985; Selkirk et al., 2005).

5.2. AChE expression and localization

The three classes of AChE have quite different tissue and cellular expression patterns. Mosaic analysis was initially used to determine that *ace-1* was expressed in muscles, and not (or very little) in neurons (Herman and Kari, 1985). Subsequent studies with reporter genes demonstrated expression in all body muscle cells, as well as the anal sphincter muscle, the four vm1 vulval muscles, the three pm5 pharyngeal muscles, and six neurons (2 OLL and 4 CEP; Culetto et al., 1999). Histochemical methods showed that the ACE-1 enzymatic activity was localized in or near the nerve ring and nerve cords and was presumably postsynaptic at neuromuscular junctions (Culotti et al., 1981).

Reporter analysis using an *ace-2* promoter indicated expression in a number of neurons in head (including the 12 IL cells and others), neurons in the tail (PVC, PVQ, and PDA), the pm5 pharyngeal muscles, and hypodermal cells near the tip of the tail (hyp8, 9, 10, 11; Combes et al., 2003). Histochemical methods showed that the ACE-2 enzymatic activity was localized near the nerve ring and nerve cords, and also near the pharyngeointestinal valve, although the cells contributing to this pattern were not identified (Culotti et al., 1981; Combes et al., 2003).

Expression of an *ace-3* reporter was observed in a few neurons in the head, a few neurons in the tail (probably PQR and PDA), the two lateral CAN cells, as well as in the pm3, pm4, pm5, and pm7 pharyngeal muscles. A dorsal row of body-wall muscle cells (the 2 dorso-medial hemiquadrants) was intensely labeled in larval stages but no longer detected in adults (Combes et al., 2003). Immunolocalization of Class C revealed staining in the nerve ring and CAN cells (there was also apparent artifactual staining of the pharyngeointestinal valve; Stern, 1986). The CAN staining in the cell processes was of a comparable intensity to the cell body, consistent with the enzyme activity being uniformly distributed in or along the plasma membrane.

It is noteworthy that although the three activities are expressed in different cell types (with the exception that all three classes are expressed in the pm5 pharyngeal muscles), all three are localized in or near the nerve ring and Classes A and B are localized in or near the ventral and dorsal nerve cords.

5.3. *ace* genes and mutants

The three *ace* genes were mapped by genetic methods and are unlinked (Johnson et al., 1981; Culotti et al., 1981; Johnson et al., 1988). Subsequently, by comparing the acetylcholinesterase homologs in the genome to the known map positions of the three *ace* loci, it was possible to clone each of the *ace* genes, analyze their transcripts and expression patterns, and characterize the available mutations (Arpagaus et al., 1994; Grauso et al., 1998; Combes et al., 2000; Combes et al., 2003). Surprisingly, in the region predicted to encode *ace-3* there were 2 AChE-encoding genes arranged in a two-gene operon (Grauso et al., 1998; Combes et al., 2003). Sequencing of an *ace-3* mutation revealed that it was in the downstream gene; although the upstream gene (now known as *ace-4*) is transcribed, the encoded protein appears to be catalytically inactive (Combes et al., 2000).

Mutations have been described for *ace-1*, *ace-2*, and *ace-3* (Johnson et al., 1981; Culotti et al., 1981; Johnson et al., 1988). Each of the single mutants is essentially wild-type, although *ace-2* animals are hypersensitive to aldicarb. The *ace-2; ace-3* and *ace-3; ace-1* double mutants are also essentially wild-type (although *ace-2; ace-3* animals are hypersensitive to aldicarb); however, *ace-2; ace-1* animals are quite uncoordinated (Culotti et al., 1981).

The *ace-2; ace-3; ace-1* triple mutant is lethal - its embryonic development is apparently normal, but many of the animals do not hatch, and those that do hatch are paralyzed and developmentally arrested (Johnson et al., 1988). A potential caveat to this phenotypic analysis is that the *ace-3* allele that was used was *dc2*, which was subsequently shown to be associated with a deletion disrupting both the *ace-3* and *ace-4* genes (Combes et al., 2000); thus the lethal phenotype is in reality associated with the quadruple *ace-2; ace-3; ace-4; ace-1* genotype. However, since the *ace-4* gene does not appear to produce a functional protein, it is likely the *ace-2; ace-3; ace-1* triple mutant would have the reported lethal phenotype.

5.4. Regulation of *ace* expression

There does not appear to be any coordinated or compensatory regulation of the *ace* genes. In each of the three single *ace* mutants and each of the three double *ace* mutants, the remaining cholinesterase activities are present at their wild-type levels, and are not elevated (Johnson et al., 1988).

6. ACh receptors - ligand gated sodium channels

The “standard” vertebrate nicotinic type of AChR is a pentameric ligand-gated anion channel. The ACh-binding α subunits may associate into homomeric structures, or they may associate with closely related non-α subunits.

The initial group of *C. elegans* receptor subunits was identified by mutations conferring resistance to levamisole (Lewis et al., 1980a; Fleming et al., 1997), but the majority of receptor subunits have now been identified by genomic sequence analysis (Mongan et al., 1998; Jones and Sattelle, 2004). A review has been published recently (Jones and Sattelle, 2004), and Table 1 summarizes the current status of this field.

Table 1. *C. elegans* acetylcholine receptor subunits.

AChR subunit	Group[a]	Type	Cellular expression[b]	Mutant phenotype	Comments	Citations
ACR-7	ACR-16	α				Mongan et al., 1998
ACR-9	ACR-16	Non-α				Mongan et al., 1998
ACR-10	ACR-16	α				Mongan et al., 1998
ACR-11	ACR-16	α				Mongan et al., 1998
ACR-14	ACR-16	Non-α	DA, DB, VB, AS, DD, HSN, VC4/5, AIY, head neurons, muscle, intestine			Mongan et al., 1998; Fox et al., 2005
ACR-15	ACR-16	α				Mongan et al., 1998
ACR-16	ACR-16	α	Body muscles, some neurons	NonUnc; but synthetic severe Unc with *unc-29* or *unc-63*	Nicotine-sensitive receptor subunit	Ballivet et al., 1996; Francis et al., 2005; Touroutine et al., 2005
ACR-19	ACR-16	α				Mongan et al., 2002
ACR-21	ACR-16	α				Mongan et al., 2002
EAT-2	ACR-16	Non-α	Pharyngeal muscles	Slow pumping		McKay et al., 2004
ACR-6	UNC-38	α				Mongan et al., 1998
UNC-38	UNC-38	α	Body and vulval muscles, many neurons	Unc, Lev	Levamisole-sensitive receptor subunit	Fleming et al., 1997; Gottschalk et al., 2005

AChR subunit	Group[a]	Type	Cellular expression[b]	Mutant phenotype	Comments	Citations
UNC-63	UNC-38	α	Body and vulval muscles, many neurons	Unc, Lev; synthetic severe Unc with *acr-16*	Levamisole-sensitive receptor subunit	Culetto et al., 2004; Touroutine et al., 2005; Ruaud and Bessereau, 2006
ACR-2	UNC-29	Non-α	VA, DA, VB, DB, IL1, RMD, and PVQ		Possible operon with *acr-3*	Squire et al., 1995; Hallam et al., 2000; Y. Jin, cited in Nurrish et al., 1999
ACR-3	UNC-29	Non-α			Possible operon with *acr-2*	Baylis et al., 1997
LEV-1	UNC-29	Non-α	Body muscles, some neurons in ventral cord	Unc, Lev	Levamisole-sensitive receptor subunit	Fleming et al., 1997; Culetto et al., 2004
UNC-29	UNC-29	Non-α	Body muscles, some neurons	Unc, Lev; synthetic severe Unc with *acr-16*	Levamisole-sensitive receptor subunit	Fleming et al., 1997; Francis et al., 2005
ACR-8	ACR-8	α	Body muscles, a few neurons in head and tail			Mongan et al., 1998; Touroutine et al., 2005; Gottschalk et al., 2005
ACR-12	ACR-8	α	Exclusively in neurons, including ventral cord neurons			Mongan et al., 1998; Gottschalk et al., 2005
LEV-8	ACR-8	α	Body muscles, uv1 and uv2 uterine muscles, PVT, ALA, many ventral cord cells including all DD cells, many head neurons, IL and OL socket cells	Lev	=ACR-13; levamisole-sensitive receptor subunit	Mongan et al., 1998; Towers et al., 2005
ACR-5	DEG-3	α	DB, VB motor neurons; other neurons in head and tail			Mongan et al., 1998; Winnier et al., 1999
ACR-17	DEG-3	α				Mongan et al., 2002
ACR-18	DEG-3	α				Mongan et al., 2002
ACR-20	DEG-3	α				Mongan et al., 2002
ACR-23	DEG-3	α				Mongan et al., 2002
DEG-3	DEG-3	α	IL2, PVD, PVC, AVG, FLP, touch cells		*deg-3* and *des-2* in operon; better response to Ch than ACh	Treinin and Chalfie, 1995; Treinin et al., 1998; Yassin et al., 2001
DES-2	DEG-3	α	IL2, PVD, PVC, AVG, FLP, touch cells		=ACR-4; *deg-3* and *des-2* in operon; better response to Ch than ACh	Treinin et al., 1998
F11C7.1	DEG-3	Non-α			Predicted, no ESTs, no data	
Y44A6E.1	DEG-3	Non-α			Several ESTs	

AChR subunit	Group[a]	Type	Cellular expression[b]	Mutant phenotype	Comments	Citations
Y73F8A.30	DEG-3	α			Predicted, no ESTs, no data	
GAR-1	GPCR		Sensory neurons in head, PVM			Lee et al., 1999; Lee et al., 2000
GAR-2	GPCR		Sensory neurons in head, ventral cord motor neurons, HSN			Lee et al., 2000; Fox et al., 2005
GAR-3	GPCR		Pharyngeal muscles, DA and/or SAB and/or I5		=ACM-3	Park et al., 2003; Steger and Avery, 2004; Fox et al., 2005
ACC-1	Ligand-gated chloride channel					Putrenko et al., 2005
ACC-2	Ligand-gated chloride channel					Putrenko et al., 2005
ACC-3	Ligand-gated chloride channel					Putrenko et al., 2005
ACC-4	Ligand-gated chloride channel					Putrenko et al., 2005
[a]The five groups of nicotinic AChR subunits are based on Mongan et al. (1998) and Jones and Sattelle (2004).						
[b]This is an incomplete list; it is likely that for many of these genes, there are additional expressing cells not listed.						

6.1. AChR subunits

It is striking that the *C. elegans* genome is extraordinarily rich in genes encoding nicotinic-type receptor subunits (Mongan et al., 1998; Jones and Sattelle, 2004). Whereas mammals and birds have fewer than 20 AChR subunit genes, genetic and genomic analysis of *C. elegans* has thus far confirmed the transcription of 27 genes (Table 1). For some of these genes, heterologous expression individually and in combination (usually in *Xenopus* oocytes) has provided information about the properties of the receptor subunits; for other genes, the identity of the ligand is merely inferred. In addition, there are about 20 genes encoding putative ionotropic receptor subunits that have not been fully characterized, but could include additional AChR subunits.

Five classes of protein subunits have been identified, based on sequence similarity (see Table 1): UNC-29, UNC-38, ACR-8, ACR-16, and DEG-3 (Mongan et al., 1998; Mongan et al., 2002; Jones and Sattelle, 2004). Each class contains three to nine members, and some of the classes contain both α and non-α type subunits. The UNC-38 class contains three α subunits, and is most similar to insect a subunits. The UNC-29 class contains four non-α subunits; these proteins are similar to vertebrate skeletal muscle non-α and insect non-α subunits. The ACR-16 class consists of nine members (α and non-α), and its members resemble vertebrate α7 subunits. The ACR-8 class is α nematode specific grouping, and contains three α subunits. The DEG-3 class is also nematode specific, and consists of eight verified α and non-α subunits (there are also two genes, one α and one non-α, predicted to be in this class, but for which there are neither ESTs nor any expression data).

The best characterized *C. elegans* AChRs, based on electrophysiological recording and reconstitution in heterologous systems, are at neuromuscular junctions. The muscles of the body wall express two major types of ACh receptors: one type responds to levamisole and the other type responds to nicotine, but not levamisole (Richmond and Jorgensen, 1999). The levamisole-sensitive receptors on the body muscles appear to be heteromeric, and contain three essential subunits: UNC-29, UNC-38, and UNC-63 (Fleming et al., 1997; Richmond and Jorgensen, 1999; Culetto et al., 2004). LEV-1 and LEV-8 are also subunits, but they are either non-essential for the response to levamisole, or else they are only present in a subset of the receptors (Fleming et al., 1997; Towers et al., 2005). The nicotine-sensitive receptors appear to be homomeric, containing only the ACR-16 α subunit (Francis et al., 2005; Touroutine et al., 2005).

For about half of the AChR subunits, expression data from reporter studies and/or antibody localization data have been reported; in general, the different subunits appear to have complex and overlapping patterns of expression. This information is presented in Table 1.

6.2. AChR genes and mutants

As might be expected, loss-of-function mutations in any of the five subunits which comprise the muscle levamisole-sensitive receptor (described above) lead to levamisole resistance (Lewis et al., 1980b). However, loss-of-function mutations in *unc-29*, *unc-38*, and *unc-63* confer strong levamisole resistance and a mild uncoordinated phenotype, while mutations in *lev-1* and *lev-8* lead to mild levamisole resistance and almost normal locomotion (Lewis et al., 1980b). In addition, *acr-16* mutations, which eliminate the function of the muscle nicotine-sensitive receptor, have essentially wild-type locomotion (Francis et al., 2005; Touroutine et al., 2005). Thus, eliminating the function of either the levamisole-sensitive receptor or the nicotine-sensitive receptor does not have profound behavioral consequences. However, eliminating the function of both receptors (e.g., an *unc-29; acr-16* double mutant or an *unc-63;acr-16* double mutant) leads to a synthetic severe uncoordinated phenotype (Francis et al., 2005; Touroutine et al., 2005).

The *deg-3* and *des-2* genes are in an operon and encode similar AChR subunits (Treinin et al., 1998). There are rare dominant mutations in *deg-3* that cause neuronal degeneration (presumably by hyperactivation of the channel); these mutations are suppressed by loss-of-function mutations in either *des-2* or *deg-3* (Treinin and Chalfie, 1995). The DEG-3 and DES-2 subunits can associate with each other to form a functional receptor *in vitro* (and presumably *in vivo*; Treinin et al., 1998). This heteromeric receptor responds more strongly to choline than acetylcholine (Yassin et al., 2001). The two genes are expressed in sensory neurons, and the DEG-3 protein is enriched in the sensory endings of these cells (Yassin et al., 2001). This suggested that the DEG-3/DES-2 receptor might play a role in sensory transduction, and *deg-3* and *des-2* mutants have been shown to be deficient in chemotaxis to choline (Yassin et al., 2001).

7. Other ACh receptors

Two additional types of ACh receptors have been identified in *C. elegans*: G-protein coupled receptors (similar to vertebrate muscarinic receptors), and ligand-gated chloride channels (which are not found in vertebrates).

7.1. GAR proteins - G-protein coupled ACh receptors

The apparent existence of muscarinic-type ACh receptors in *C. elegans* was first reported in 1983 (Culotti and Klein, 1983), and subsequently verified through analysis of the *gar-1*, *gar-2*, and *gar-3* genes. The primary transcripts from all three of the *gar* genes undergo alternative splicing (Park et al., 2000; Suh et al., 2001; Park et al., 2003), leading to considerable protein diversity. Pharmacological profiles indicate that the GAR-3 isoforms are the most similar to “conventional” vertebrate muscarinic receptors, and bind the antagonist scopolamine and the agonist carbachol (Hwang et al., 1999; Park et al., 2003). The GAR-1 isoforms are somewhat different, and bind atropine, but not scopolamine (Lee et al., 1999), while GAR-2 binds neither atropine nor scopolamine (Lee et al., 2000). Complete expression profiles for these receptors have not been reported. However, GFP constructs indicated that *gar-1* and *gar-2* are expressed in neurons, including some sensory neurons in the head, PVM (*gar-1* only), motor neurons in the ventral cord (*gar-2*), and HSN (*gar-2*) (Lee et al., 2000). *gar-3* is expressed in pharyngeal muscle (Steger and Avery, 2004).

7.2. ACC proteins - ACh-gated chloride channels

The four ACC proteins are most closely related to the *C. elegans* MOD-1 serotonin-gated chloride channel, and have no orthologs in vertebrate or *Drosophila* genomes (Putrenko et al., 2005). ACC-1 can form a homomeric ACh-gated chloride channel with an EC_{50} of 0.26 μM, or a heteromeric channel together with ACC-3, with an EC_{50} of 39.6 μM (Putrenko et al., 2005). ACC-2 can also form homomeric channels, with an EC_{50} of 9.54 μM. Nothing has yet been reported about the expression patterns of these proteins, or mutant phenotypes.

8. Cholinergic receptor-associated proteins and genes

Several proteins have been described that seem to be associated with cholinergic function and are required for the expression, maturation, trafficking, and/or localization of particular ACh receptor subunits.

RIC-3 (a protein containing two transmembrane domains followed by three coiled-coil regions) is required for maturation of at least four types of ACh receptor: the EAT-2-containing pharyngeal AChR, the DEG-3/DES-2 neuronal AChR, the UNC-29-containing levamisole-sensitive muscle AChR, and the ACR-16-containing nicotine-sensitive muscle AChR (Halevi et al., 2002). RIC-3 is expressed in pharyngeal and body wall muscles, and in most or all neurons, and is localized to cell bodies. *ric-3* mutants are Unc, Ric, and Lev (Nguyen et al., 1995; Miller et al., 1996).

LEV-10 (a transmembrane protein containing five extracellular CUB domains) is required for the clustering of post-synaptic UNC-29-containing AChRs (Gally et al., 2004). Consistent with this role, LEV-10 is expressed in body muscle, and is localized to post-synaptic cholinergic neuromuscular junctions (Gally et al., 2004).

EAT-18 (a small transmembrane protein) is somehow required for proper function of pharyngeal AChRs containing the EAT-2 non-α subunit, and apparently other pharyngeal nicotinic receptors as well (McKay et al., 2004). Surprisingly, the *eat-18* gene and promoter are nested within the first intron of *lev-10* (Gally et al., 2004). *eat-18* mutants resemble *eat-2* mutants, demonstrating the importance of EAT-18 for EAT-2 function (McKay et al., 2004).

CAM-1 (a Ror receptor tyrosine kinase) is required for the localization and clustering of ACR-16-containing nicotine-sensitive muscle AChRs (Francis et al., 2005), and also plays a role in the localization or function of the levamisole-sensitive receptors, but it is not involved in the localization or function of GABA receptors (Gottschalk et al., 2005). CAM-1 is expressed in muscles and is enriched in the muscle arms and especially at neuromuscular junctions. CAM-1 is also expressed in many neurons, including ventral cord cholinergic motor neurons. Analysis of *cam-1* mutants shows that the protein is also involved in cell migration and axon guidance (Forrester et al., 1999).

Neither *unc-50* nor *unc-74* encodes an AChR subunit, yet mutations in these two genes lead to strong resistance to levamisole (Lewis et al., 1980b). Therefore, the UNC-50 and UNC-74 proteins must be required in some way for receptor response to levamisole (Lewis et al., 1980a). Although molecular analysis of neither *unc-50* nor *unc-74* has been reported directly, indirect reports indicate that the *unc-50* gene encodes a novel type of transmembrane protein with identifiable mammalian and plant homologs.

There are also some proteins that clearly contribute to the stability, trafficking, and/or clustering of AChR subunits, but to a lesser extent than the gene products described above, and whose loss-of-function phenotypes are associated with partial reduction in the abundance of localized receptors. For example, NRA-1 is a copine localized to plasma membranes necessary for high-level expression of UNC-38 and LEV-1 (Gottschalk et al., 2005).

9. ACh-mediated behaviors

9.1. Locomotion

This is the most important acetylcholine-mediated behavior, involving by far the greatest number of cholinergic neurons. Locomotion (both crawling on surfaces and swimming in liquid) requires the ability to generate smooth, sinusoidal waves (at variable propagation rates) of either anterior-directed or posterior-directed body muscle contractions. The involvement of ACh in locomotion includes not only neuromuscular transmission, but also nerve-nerve transmission (which is poorly characterized). Thus, for example, ventral nerve cord cholinergic motor neurons express the ACR-2 and ACR-5 AChR subunits (Winnier et al., 1999; Hallam et al., 2000), while GABAergic motor neurons express LEV-8 (Towers et al., 2005). In addition, ACh appears to be involved in the regulation of the oscillator circuit controlling the rate of wave initiation: in addition to their uncoordinated movement, cholinergic (i.e., *cha-1* and *unc-17*) mutants have a dramatically reduced rate of wave initiation.

9.2. Egg laying

Egg laying behavior involves the action of several neurotransmitters (see Egg-Laying), and ACh appears to affect egg laying through multiple mechanisms. The major effect of ACh appears to be an inhibition of egg laying, because animals deficient in cholinergic release (e.g., *cha-1* and *unc-17* mutants) are constitutive for egg-laying, and retain very few embryos (Bany et al., 2003). However, levamisole can stimulate egg laying (Kim et al., 2001), suggesting a possible stimulatory cholinergic mechanism operating through levamisole-sensitive receptors on muscles.

The egg-laying muscles are innervated by the HSN and VC cells; the VC cells also make synapses onto the HSNs (White et al., 1986). A plausible model is that ACh is released by VC cells onto the presynaptic terminals of the HSN cells, and interacts with inhibitory GAR-2 (and perhaps other) cholinergic receptors (Bany et al., 2003). This leads to decreased HSN release of serotonin. This model is complicated somewhat by the apparent multi-transmitter phenotype of both the HSNs and VC4/VC5 (Duerr et al., 2001).

9.3. Pharyngeal pumping

The MC neuron is cholinergic and stimulates the pharynx muscle (via the EAT-2 receptor) to control pharyngeal pumping rate (McKay et al., 2004). Wild-type animals pump >250/min; pumping for animals ablated for MC is ~ 45/min (Avery and Horvitz, 1989; Raizen et al., 1995). It is likely that the slow growth rates of *cha-1* and *unc-17* mutants (Rand and Russell, 1984) are related to their reduced rate of pharyngeal pumping.

In addition, a number of other ACh receptor types are expressed on pharyngeal cells, and appear to mediate several aspects of pharynx function. For example, the muscarinic GAR-3 receptor, signaling through EGL-30 and GPB-2, is involved in control of pharyngeal muscle membrane potential (Steger and Avery, 2004).

9.4. Defecation cycling

Mutations in *cha-1* (Thomas, 1990) and *unc-17* (J. Rand, unpublished) cause dramatically longer and variable defecation cycle periods. However, when defecation does occur, it appears to be normal (Thomas, 1990). It is not known which cholinergic neurons are responsible for regulating this periodic behavior.

9.5. Male mating

Several distinct cholinergic pathways in the male tail have been described that are involved in male mating behavior (see Male mating behavior). The PCB and PCC sensory neurons of the postcloacal sensilla are cholinergic, and together with the anal depressor and some still unidentified cells, mediate periodic contractions (“prodding” behavior) of the protractor muscles (Liu and Sternberg, 1995; Garcia et al., 2001). The subsequent spicule insertion requires prolonged contraction of the protractor muscles, mediated by the cholinergic SPC motor neurons. Protractor contraction (both the periodic prodding and the prolonged contraction) is severely deficient in *cha-1* mutants, and is potentiated in wild-type animals by aldicarb; the cholinergic signaling is mediated by multiple receptors, including distinct levamisole-, nicotine-, and arecoline-sensitive receptors (Garcia et al., 2001).

10. Additional aspects of cholinergic biology

10.1. Transmitter coexpression

Although we are used to thinking that each neuron releases a single neurotransmitter, this turns out to be an oversimplification. For example, the four motor neurons with significant output to the egg-laying muscles, HSNL/R and VC4/5 appear to release both ACh and serotonin (Duerr et al., 2001). It is not known if the two transmitters are colocalized in the same vesicles, or if they are released from the same synapses.

10.2. Role in protein turnover

One of the consequences of starvation is the triggering of protein degradation in muscles. This may be monitored as the disappearance of muscle reporter expression in response to starvation (Zdinak et al., 1997). The starvation-dependent proteolysis is negatively regulated by cholinergic input to the muscles (Szewczyk et al., 2000). For example, mutants deficient in ACh release or reception (e.g., *cha-1*, *unc-17*, *unc-13*, *unc-38*) have enhanced starvation-induced protein turnover, while the cholinergic agonist levamisole completely prevents starvation-induced turnover in muscle cells (Szewczyk et al., 2000).

10.3. Cholinergic regulation of developmental timing

Ruaud and Bessereau (2006) have reported that application of the nicotinic receptor agonist DMPP slowed overall *C. elegans* development during the L2 stage but did not affect the molt timing, so that the L2/L3 molt occurred before the L3 cuticle was ready, and the animals died. This suggests the uncoupling of a “developmental timer” apparently regulated by cholinergic signaling from a “molting timer”. The action of the drug was not due to

general toxicity, but rather was specific and was mediated through UNC-63-containing receptors, because *unc-63* mutants were partially resistant to the stage-specific lethality of DMPP. However, the DMPP sensitivity was not mediated by the muscle levamisole-sensitive receptors, but rather by neuronal UNC-63-containing receptors. The specific neuronal pathways mediating this effect have not yet been determined.

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