Genetics of Neurotransmitter Release in *Caenorhabditis elegans*☆

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Glossary

Endocytosis The removal of lipids and proteins from the plasma membrane by internalization of membrane vesicles.
Exocytosis The process in which a cell releases the contents of a secretory vesicle into the extracellular space by fusing the vesicle membrane with the plasma membrane.
Genetic screen A procedure to identify individuals in a population with a mutant phenotype of interest.
Nematode An unsegmented pseudocoelomate, also referred to as a roundworm. One of the most diverse phyla with over 25,000 species described.
Transgenic animal An individual in which an exogenous piece of DNA (a transgene) has been introduced into the genome.

1 Using a Nematode to Study Neurobiology

In 1974 Sydney Brenner introduced *Caenorhabditis elegans* as a genetic model organism that could be used to elucidate the molecular nature of the nervous system. The main advantages of *C. elegans* as a model organism for the study of genetic pathways in general include the simplicity of worm maintenance, the ease of isolating mutants, and the availability of molecular reagents for gene analysis. In addition, the nematode possesses a number of features that make it particularly well-suited for the study of its nervous system. First, the nervous system – comprised of only 302 neurons – is small and invariant between individuals (Fig. 1), and the connectivity of the nervous system has been reconstructed from serial electron micrographs. Second, the worm is transparent, so individual cells can be identified using a light microscope and individual synapses can be imaged using fluorescence microscopy. Third, the nervous system is largely nonessential under laboratory conditions. Locomotion is not required since the animals are grown on bacteria, their food. This nematode ingests the bacteria using a muscular pump called the pharynx, and the pharynx will pump even in the absence of neuronal input. Hence mutants like *unc-13* that lack synaptic transmission are viable. However, laser ablation studies have demonstrated that there are two neuron types, M4 and CAN, which are required for viability and animals that lack both fast and modulatory neurotransmission are probably dead. Nevertheless, because the worm is so robust, mutants with severe defects in the nervous system can be maintained and studied. Fourth, one sex in *C. elegans* is a self-fertilizing hermaphrodite so it does not need a nervous system to search for mates and to reproduce; a single individual – even if it is paralyzed – can be moved to a fresh plate to propagate the strain. Males can be used to cross strains together to produce complex genotypes.

Recent advances in the technology to produce transgenic *C. elegans* have made targeted gene knockouts and structure-function studies possible. Previously, transgenes were maintained as heritable extrachromosomal arrays comprised of hundreds of copies of the injected DNA. Multicopy transgenes can result in overexpression, dominant negative effects, toxicity, promoter titration or gene silencing. Some tissues, such as the germline or muscle, are refractory to expression from arrays. In addition, the arrays are unstable during mitosis so that individual transgenic animals are mosaic, and are unstable during meiosis so that the array is lost in subsequent generations. A number of technologies for modifying the genome have arisen that allow the modification of the genome at will. A particular sequence can be inserted into the genome, by introducing a double-strand break at a particular site

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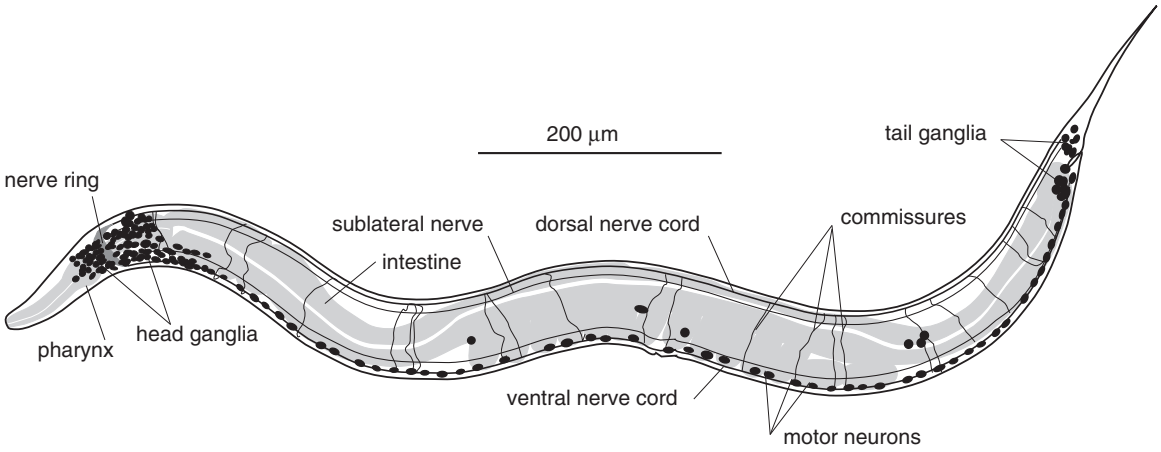


Fig. 1 The adult nervous system of *Caenorhabditis elegans*. There are 302 neurons in an adult nematode. Most of the cell bodies of the neurons are found in the head ganglia, in the ventral nerve cord or in the tail ganglia. Not all axon bundles are shown.

and a DNA template provided to heal the break. There are two methods: MosSCI, which inserts modified genes at a new locus, and CRISPR, which modifies the endogenous locus.

MosSCI uses the Mos1 transposon, a fly transposon that has been introduced into the worm. A consortium of French scientists has generated a library of tens of thousands of *C. elegans* strains, each containing a Mos element at a different site in the genome. Excision of a transposon introduces a double-strand break in the DNA, which can be used to modify the site in several ways, for example deleting adjacent genes (MosDEL), modifying a gene (MosTIC), or introducing a single copy of a transgene (MosSCI). These technologies are used for protein tagging with fluorescent proteins, to determine tissue specificity of a gene, or to introduce structural variants into proteins.

The CRISPR/Cas9 system has become the standard genome modification technique for nearly all model organisms and has proved to be extremely simple to implement in *C. elegans*. The Cas9 protein cuts genomic DNA sequences that are targeted by a 20 nucleotide RNA sequence. A DNA template is co-injected that overlaps the broken ends of the chromosome but also includes inserted DNA or changes to the original sequence. These changes to the DNA are then incorporated into the genome when the break is healed. CRISPR is especially useful for deleting or modifying genes in situ.

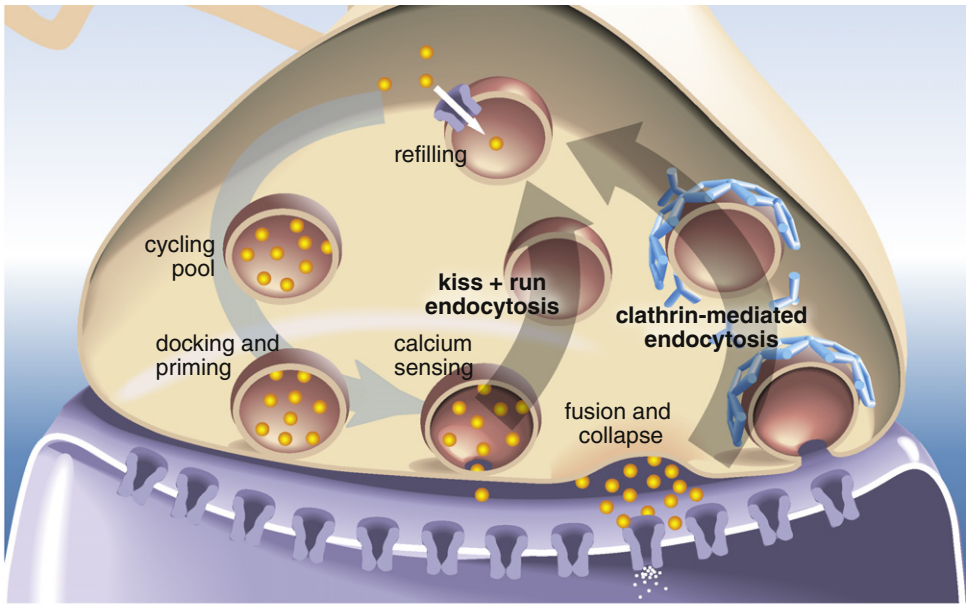
2 Genetic Dissection of Neurotransmission

Communication between neurons is mediated by neurotransmitters. When a neuron is depolarized, calcium enters the neuron via voltage-sensitive calcium channels, calcium then causes the synaptic vesicles to fuse with the plasma membrane and release neurotransmitter into the synaptic cleft. Once the vesicle has released its contents, the vesicle and its associated proteins are retrieved from the membrane and prepared for another cycle of fusion (**Fig. 2**). Biochemical studies of yeast and mammalian cells have identified many of the proteins required for vesicle dynamics. Genetic studies in *C. elegans* have identified additional components and have also elucidated the functions of these proteins at the synapse. The proteins uncovered in these genetic studies can be divided into two categories: those required for a specific neurotransmitter type, and those required for the functions of all synapses, for example, proteins required for synaptic vesicle kinetics. We will highlight those synaptic components, either neurotransmitter specific or general, that were discovered in *C. elegans*.

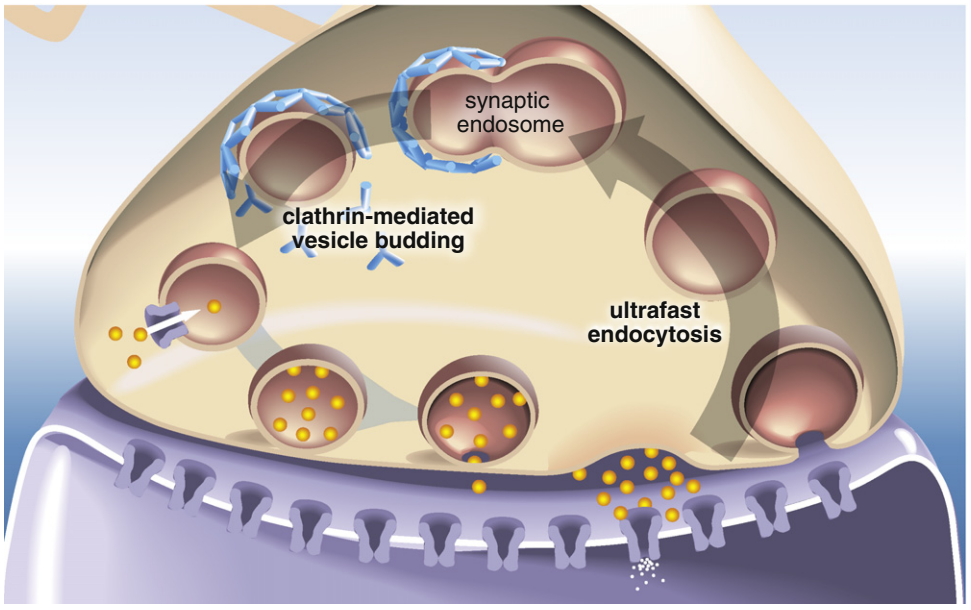
3 Neurotransmitters

The original genetic screens for synaptic function involved the identification of mutant *C. elegans* that had difficulties moving. While these screens identified some of the genes involved in synaptic transmission, it was difficult to tease these apart from genes required for other nervous system functions, such as development. To determine the molecules specifically involved in neurotransmitter release required the use of more focused screens. We will focus on the two best-studied neurotransmitters, GABA and acetylcholine, the two major neurotransmitters at the *C. elegans* neuromuscular junction.

Loss of all GABA neurons by laser ablation of the cell bodies leads to a very specific uncoordinated phenotype. When a worm lacking the GABA nervous system is tapped on its nose it shrinks. Forward genetic screens for shrinker mutants which mimicked the loss of GABA, identified five of the seven genes required for GABA function (**Fig. 3**). The proteins encoded by these genes include: UNC-25, the biosynthetic enzyme for synthesizing GABA, UNC-47, the vesicular GABA transporter, UNC-46, a LAMP family protein, UNC-49, a GABA receptor, and UNC-30, a transcription factor. The UNC-46 protein (the mammalian homolog is called LAMP5/BAD-LAMP) is required for localization of the GABA transporter, UNC-47, to synaptic vesicles. UNC-30 is a



(a)



(b)

Fig. 2 The synaptic vesicle cycle. Neurotransmitter (yellow) is loaded into synaptic vesicles. The vesicles dock and become primed for exocytosis. Calcium influx acts through a calcium sensor to stimulate fusion of the vesicle and plasma membranes. After fusion, membrane and vesicle proteins are recycled by endocytosis. Vesicles are refilled and re-enter the cycling pool. (a) Conventional endocytosis. Until recently the bulk of endocytosis has been thought to be via clathrin-mediated reformation of a synaptic vesicle at the plasma membrane. There is some evidence that synaptic vesicles do no fully collapse into the plasma membrane but could rather regenerate themselves by closing the fusion pore in a process known as "kiss and run". (b) Ultrafast endocytosis. Recently a rapid mechanism for recycling synaptic vesicles has been discovered in *Caenorhabditis elegans* that can remove membrane within 30–50 ms.

homeodomain transcription factor required for specification of neurotransmitter type in GABA neurons. Several of these molecules were subsequently identified in vertebrates as important for GABA signaling. In addition, novel excitatory GABA receptors were identified using a more specific behavioral screen. These GABA-gated cation channels, EXP-1 and LGC-35, are found in nematodes but not other phyla. The GABA metabotropic receptors GBB-1 and GBB-2, and the plasma membrane transporter SNF-11 were not identified in forward screens, but rather were identified by molecular similarity. The plasma membrane transporter SNF-11 is not required for GABA function, presumably because GABA synthesis can provide sufficient neurotransmitter in the absence of recovery by reuptake.

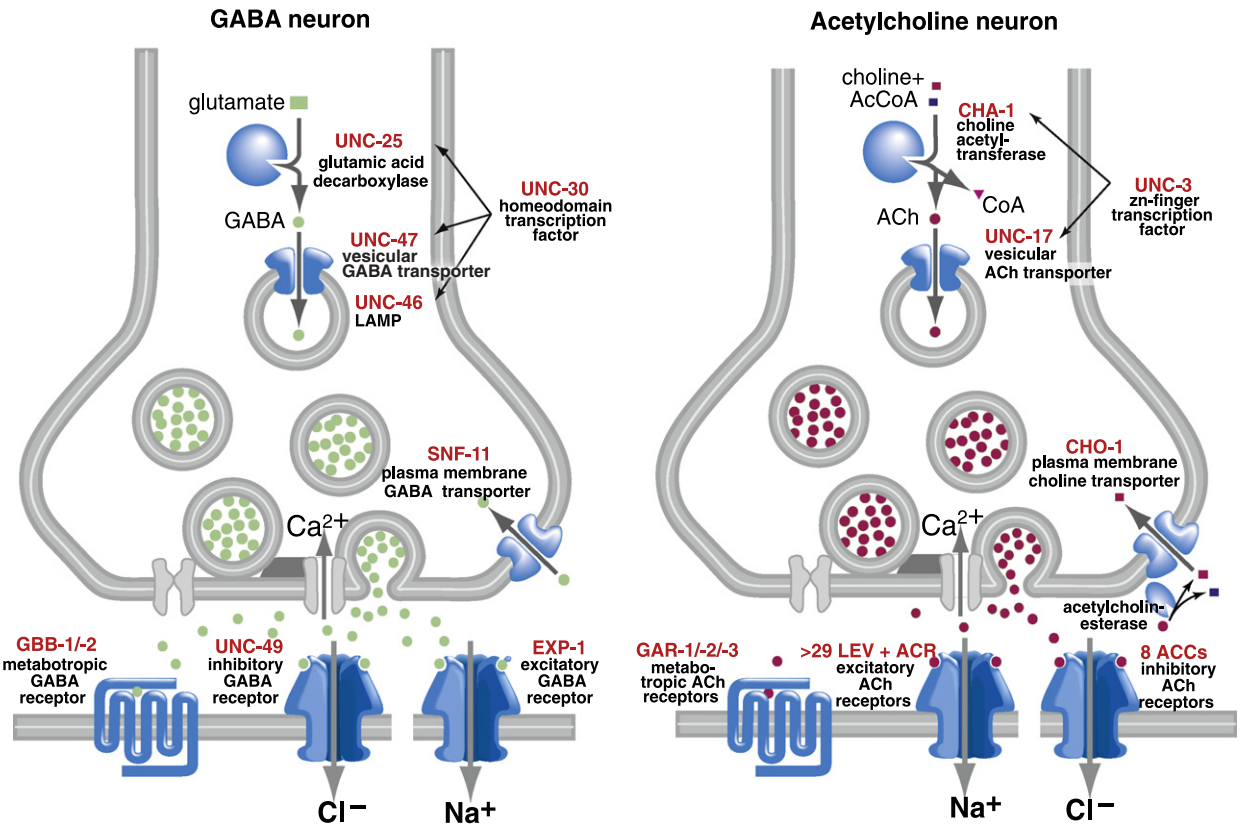


Fig. 3 Neurotransmitter-specific molecules for GABA and acetylcholine neurotransmitters identified in genetic screens. GABA mutants were identified because they resembled animals with GABA neurons ablated. Loss of acetylcholine transmission can be identified by screening for mutants that fail to release acetylcholine (resistant to acetylcholinesterase inhibitor aldicarb) or fail to sense acetylcholine (resistant to receptor agonist levamisole). Other receptors were identified by protein homology. Protein functions (black) and *Caenorhabditis elegans* 3-letter protein names (red) are shown. GABA = Gamma aminobutyric acid. ACh = Acetylcholine. AcCoA = Acetyl CoA.

Loss of acetylcholine neuron function leads to a lethal phenotype. However, it has been still possible to identify weak alleles of the genes involved in acetylcholine transmission due to their resistance to acetylcholinesterase inhibitors. The most commonly used is Aldicarb, a potent inhibitor of acetylcholinesterases at the neuromuscular junction. Exposure to Aldicarb blocks the active site of the acetylcholinesterase enzyme and leads to accumulation of acetylcholine in the synaptic cleft. The accumulating acetylcholine leads to hypercontraction of the body muscles and a constitutively open pharynx which eventually leads to death of the animal – perhaps due to a loss of osmoregulation (Fig. 4). Therefore it is possible to identify mutants with either decreased or increased acetylcholine release by screening for mutants that are resistant or hypersensitive, respectively, to Aldicarb. Screens designed to measure for altered levels of acetylcholine release led to the identification of *cha-1*, the biosynthetic enzyme required for acetylcholine synthesis and *unc-17*, the vesicular acetylcholine transporter. Although Aldicarb sensitivity is a measure of levels of acetylcholine release, resistant mutants are not necessarily specific to acetylcholine transmission. Any mutation that affects the machinery of neurotransmitter release or synaptic development will also give rise to Aldicarb resistance. Thus Aldicarb has been an extremely useful tool for understanding acetylcholine transmission, but it has also been useful to identify proteins involved in general synaptic function.

One of the near misses in *C. elegans* genetics was the identification of the family of ligand-gated ion channels. Mutants lacking acetylcholine receptors were characterized by Jim Lewis in 1980, because they are resistant to the agonist levamisole. However, at this time molecular techniques were not advanced in *C. elegans* and the gene sequences could not be characterized. Two years later, acetylcholine receptors from the electric ray *Torpedo* were cloned and characterized in the laboratories of Numa, Heinemann and Changeux. Ironically, when the genes encoding acetylcholine receptors in *C. elegans* were identified it was discovered that the genome is particularly rich in acetylcholine subunits – there are at least 29 genes encoding subunits of acetylcholine receptors, and others that need to be validated.

C. elegans has also provided some surprises in the diversity of neurotransmitters it uses. The worm has the full repertoire of classical transmitters and peptides; however, a novel neurotransmitter regulates the defecation cycle. The cycle is a 50-second program comprised of a posterior body contraction followed by enteric muscle contractions which expel the contents of the gut. Contraction of the posterior body muscles is regulated by an intestinomuscular synapse. The neurotransmitter at the junction is a subatomic particle: the proton. Release of protons from the intestinal cell at synapses is mediated by PBO-4, a sodium proton

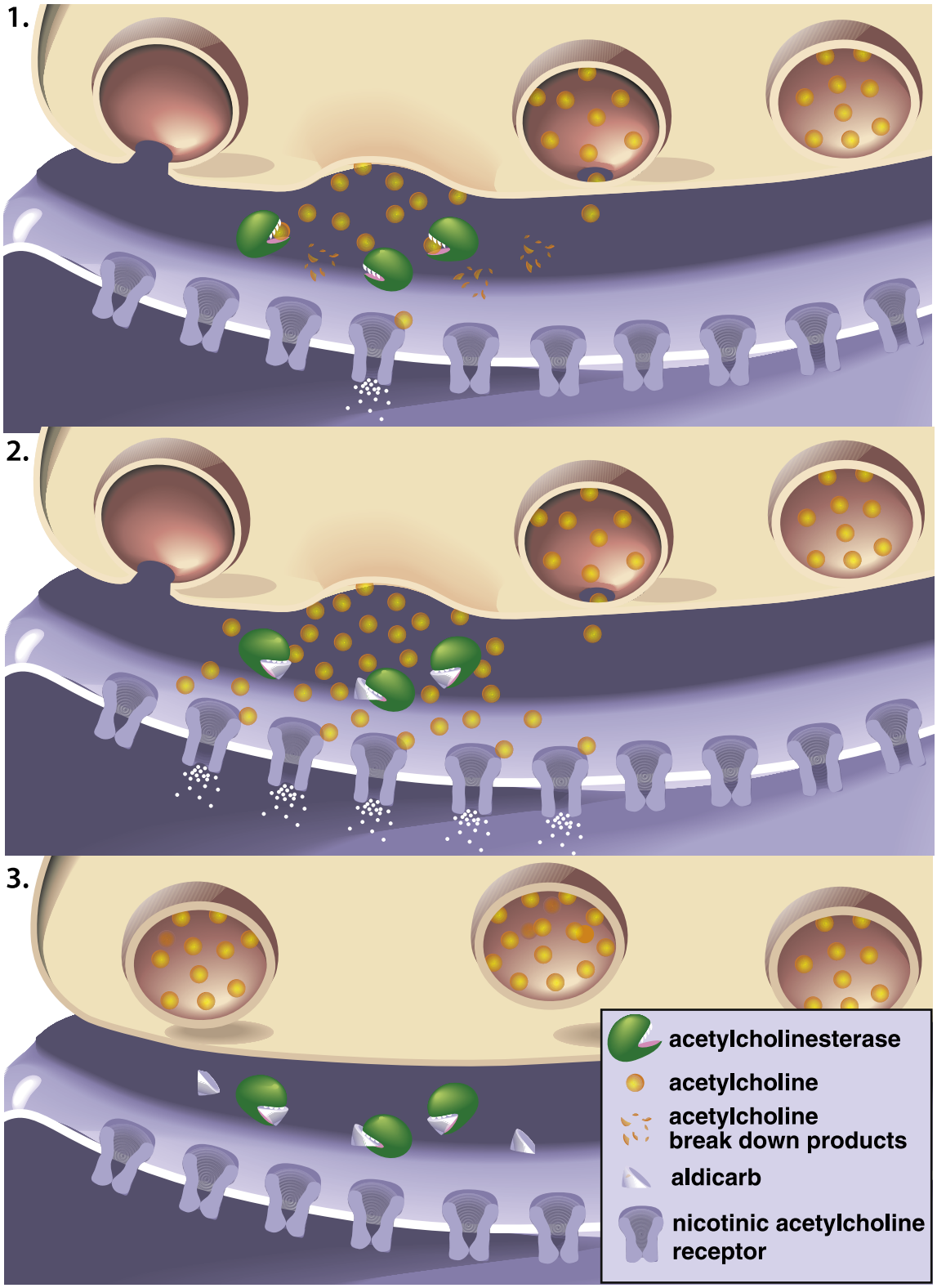


Fig. 4 Aldicarb blocks acetylcholine degradation. Above, in a wild-type synapse, acetylcholine is rapidly degraded by acetylcholinesterase to terminate transmission and reset the synapse. Middle, Aldicarb blocks acetylcholinesterase and acetylcholine accumulates in the cleft, which leads to chronic activation of the neuromuscular junction and contraction of the muscle. Hypercontraction of the muscle and death of the animal is not caused directly by Aldicarb but rather by the natural release of acetylcholine at the synapse. Below, in mutants with defects in exocytosis such as *unc-13*, the synapse releases little or no acetylcholine and the animal is resistant to Aldicarb.

exchanger. The proton receptor on the muscles is comprised of the PBO-5 and PBO-6 subunits, which resemble acetylcholine-gated ion channel subunits. Export of protons into the synaptic cleft activate this cation channel and cause muscle contraction. PBO-5 is also expressed in the nervous system suggesting protons could also be functioning as signaling molecules within the nervous system.

4 Trafficking of Vesicles

Proteins required for the functioning of all synapses include proteins required to transport materials from the cell body to the synapse and proteins required to dock, fuse and recycle synaptic vesicles at the active zone. The cell body of the neuron is often far from the synapse. To transport synaptic vesicle precursors to the synapse neurons use a kinesin-like motor protein encoded by *unc-104*. Worms with reduced function of *unc-104* accumulate vesicle precursors in the cell body. Vertebrate homologs have been discovered and comprise the Kinesin-3 family of kinesins.

5 Synaptic Vesicle Cycle

The synaptic vesicle cycle has a number of stages. A synaptic vesicle precursor is filled with neurotransmitter, docks with the plasma membrane and then primes to become competent for fusion. A primed vesicle then fuses with the plasma membrane to release its neurotransmitter into the synaptic cleft upon calcium influx. The synaptic vesicle proteins and membrane are subsequently recycled and the vesicle reformed by endocytosis (Fig. 2).

6 Exocytosis

The fusion of synaptic vesicles with the plasma membrane requires the formation of the SNARE complex (Fig. 5). The SNARE complex is comprised of three proteins: syntaxin/UNC-64, SNAP-25 and synaptobrevin/SNB-1. Both syntaxin and SNAP-25 are associated with the plasma membrane, whereas synaptobrevin is an integral membrane protein of the synaptic vesicle. These three proteins form a helical bundle which pulls the vesicle close to the plasma membrane at the active zone which is thought to induce membrane fusion. The SNARE complex is absolutely required for vesicle fusion. Null mutations in syntaxin and synaptobrevin abolish synaptic vesicle release. Two proteins implicated in regulating the formation of the fusion complex are UNC-18 and UNC-13. The discovery of *unc-18* and *unc-13* in *C. elegans* led to the identification of homologs in vertebrates. Null mutations in either one of these proteins result in a severe decrease in neurotransmission. Both proteins have been demonstrated to bind to syntaxin. In the absence of UNC-18 there is a severe decrease in the release of synaptic vesicles; thus it must be playing a facilitatory role in neurotransmission. The N-terminus of syntaxin recruits UNC-18 to the SNARE bundle to assembly fusion-competent SNARE complex.

In animals with reduced UNC-13 function, neurotransmitter release is abolished. UNC-13 converts the SNARE syntaxin from a closed configuration into an open configuration so that it can engage the SNAP-25 and the vesicular SNARE protein synaptobrevin, and thereby generates a docked and primed vesicle. UNC-13 is the target of modulatory cascades which increase

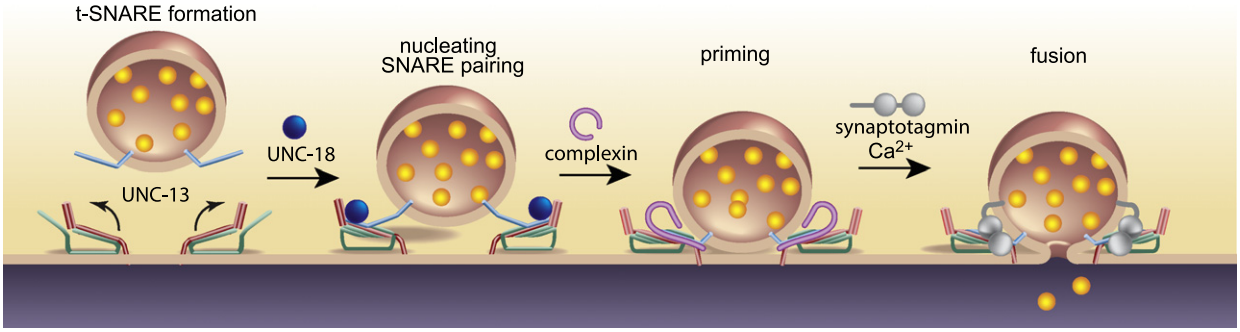


Fig. 5 Steps in exocytosis. Regulated fusion of the vesicle and plasma membranes requires several steps. First, the target SNARE complex, composed of syntaxin (red) and SNAP-25 (green) must form; this is likely achieved by the conversion of syntaxin to the open conformation by UNC-13. Second, the trans-SNARE complex must be nucleated by the vesicular SNARE synaptobrevin interacting with the target-SNAREs; this may be achieved in part by UNC-18 which has a post-docking role. Third, after docking the vesicle must be primed for fusion. Priming requires an additional protein complexin (purple) and likely involves the full intertwining of the SNARE proteins. Fourth, the calcium sensor, synaptogmin becomes engaged, and upon calcium influx stimulates fusion of the vesicle with the plasma membrane, resulting in the release of neurotransmitter into the synaptic cleft.

neurotransmission. Specifically, UNC-13 acts in a G-protein signaling pathway downstream of Gq alpha/EGL-30 and phospholipase-C/EGL-8. Activation of these modulatory pathways stimulates UNC-13 and thereby increases vesicle priming.

Once the vesicle is primed fusion is triggered by the influx of calcium via ion channels. Two proteins play an essential role in triggering fusion in response to fusion, complexin and synaptotagmin. Complexin, or CPX-1, in *C. elegans* acts as a brake to prevent the premature fusion of synaptic vesicles. In the absence of complexin leads to the release of more neurotransmitter, but also a decrease in the number of docked vesicles. Complexin is a small, ~130 amino acid protein that binds to the SNARE complex. The C-terminal tail of complexin recruits complexin to synaptic vesicles. Its central helical domain is required for binding to the SNARE complex and is essential for function, and the N-terminus of the protein is essential for complexin's function as a brake. When the *C. elegans* CPX-1 accessory helix domain was replaced with a completely unrelated helical peptide it was capable of rescuing the mutant suggesting that it is in fact the presence of a helix that is most important for complexin's function and not the sequence itself.

The Ca^{2+} sensor is synaptotagmin/SNT-1. Synaptotagmin is an integral membrane protein of the synaptic vesicle, contains two C2 Ca^{2+} binding domains and has been demonstrated to bind to the SNARE complex. Absence of synaptotagmin causes a loss of fast calcium-dependent neurotransmitter release in mice. Analysis of synaptotagmin's role in *C. elegans* is complicated by its role in endocytosis.

7 Endocytosis

Once a vesicle fuses with the plasma membrane, the synaptic vesicle and its associated proteins are retrieved from the plasma membrane by endocytosis (Fig. 6). Synaptic vesicle endocytosis was originally thought to be mediated by the formation of a clathrin cage which buds membrane into the cell. Although clathrin-mediated endocytosis undoubtedly occurs at some synapses, at *C. elegans* neuromuscular junctions a fast form on endocytosis may predominate. Upon stimulation vesicles are fused with the plasma membrane across a broad active zone at the *C. elegans* neuromuscular junctions. Thereafter, large endocytic vesicles (2–3 times the size of a synaptic vesicle) bud off from the plasma membrane at the dense projection and at the edge of the active zone at the adherens junctions. These endocytic events also happen much faster than 30 s previously observed for clathrin-mediated endocytosis: Endocytosis at the dense projection occurs between 30–50 ms and at the adherens junction between 1–3 s. Finally, 'ultrafast endocytosis', which was originally discovered in *C. elegans*, is conserved in vertebrates.

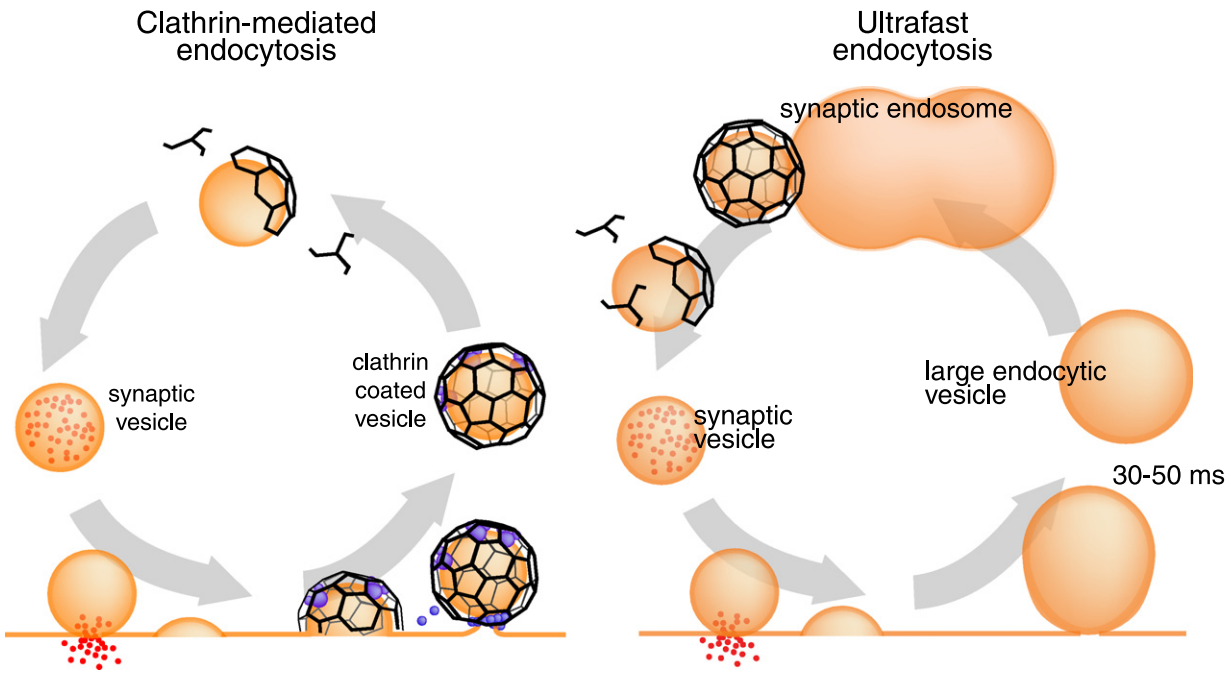


Fig. 6 Alternative models for synaptic vesicle endocytosis. Left panel. Clathrin-mediated endocytosis, the classical model for synaptic vesicle recycling. After vesicle fusion a clathrin coat reforms a synaptic vesicle on the plasma membrane resulting in a clathrin-coated vesicle in the cytosol of the neuron. The vesicle is uncoated and develops into a fusion-competent vesicle for another round of fusion. Right panel. Ultrafast endocytosis, an alternative model for synaptic vesicle cycling. Here after a round of vesicle fusion a large endocytic vesicle is invaginated from the plasma membrane and cleaved by dynamin. These large vesicles may fuse to form a synaptic endosome. Clathrin is used to regenerate synaptic vesicles from the endosome which can then be uncoated, refilled with neurotransmitter and become fusion competent.

Synaptic vesicle proteins are recruited to endocytic sites by specific adaptors. For example, the clathrin adaptor protein AP180/UNC-11 recruits the synaptic vesicle protein synaptobrevin during endocytosis. The mu homology domain of stonin/UNC-41 and the mu homology domain of the clathrin adaptor AP2 bind the C2 domains of the synaptic vesicle protein synaptotagmin. However, it is possible that in the case of synaptotagmin that it is acting to recruit these factors to nuclear endocytic sites rather than vice versa.

Membrane invagination requires changes in the shape and composition of the membrane. The BAR domain protein endophilin/UNC-57 is likely to bind and curve the membrane. And endophilin is required to recruit the lipid phosphatase synaptojanin. Synaptojanin/UNC-26 a polyphosphoinositide phosphatase that converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol (PI). Thus, the lipid composition of synaptic membranes plays an important role in regulating progress through endocytosis. The invaginating pit is then cleaved from the plasma membrane by dynamin, encoded by *dyn*-1. Eventually the synaptic vesicle is reformed inside the synaptic bouton, mostly likely by clathrin. To complete vesicle recycling the clathrin coat must be removed. Mutations in both synaptojanin and endophilin result in an accumulation of coated vesicles, presumably because the adaptor proteins which bind PIP2 remain attached to synaptic vesicle lipids.

8 Propagation of the Electrical Signal Along Axons

In the mammalian nervous system most information is coded by the frequency of action potentials. *C. elegans* lacks action potentials for the most part, and in fact lack the voltage-gated sodium channels which are essential components of all-or-none action potentials. In *C. elegans* signal propagation along the axon appears to be via graded transmission much like in vertebrate photoreceptors and auditory sensory neurons. Graded synaptic transmission is a passively propagated electrical signal. Although regenerative currents can be identified in some neurons such as the RMD motor neuron in the head, neurotransmitter release from the main motor neurons in the ventral cord in *C. elegans* is tonic, that is, the rate of release of synaptic vesicles seems to be proportional to the membrane potential of the motor neuron.

9 Future

The *C. elegans* genome contains 20,043 genes and currently >90% have a potentially null mutation; the hard work is to assign a function to these genes. The *C. elegans* genome, of course, is the most important resource for further study and more sophisticated screens or studies of novel biological processes will reveal the function of these genes. However, forward genetic screens suffer from two drawbacks common to all genetic studies: redundancy and lethality.

A number of loci exhibit genetic redundancy, that is, a phenotype is only observed when multiple genes are mutated. These have usually been identified by luck: a mutant strain was identified with mutations in each of the redundant genes. On the other hand, if there are *a priori* reasons to believe that redundant pathways might exist, then enhancer screens can identify genes in these parallel pathways. Strains carrying mutations in a gene in the redundant pathway can be mutagenized to identify second-site mutations that exhibit synthetic phenotypes. The drawback with such screens is that the double mutants will be sicker than the starting strain, or even lethal. Although there are ways to circumvent these problems, the solutions involve complicated genetic backgrounds, and thus such screens are rarely performed.

To solve the issue of lethality techniques are being developed that generate mosaic animals, in which a gene is mutant in a particular cell in an animal that is wild-type in all other cells. CRISPR can be used to introduce site-specific recombination sites for FLP or Cre into an endogenous locus. The recombinase can then excise the gene when the enzyme is expressed in a specific cell, and the phenotype identified in this single cell. Again, these are laborious screens.

On the other hand, suppressor screens are the strength of *C. elegans* forward genetic screens. Particularly advantageous for such screens is hermaphroditic reproduction. This means that a population of mutant worms can be mutagenized and double mutants selected for improved health. In this case lethality can work for the geneticist: suppressor screens can be used to identify mutations that restore viability. At first glance it is bewildering that such screens are possible: if removing a single gene from an animal makes it very sick, how is it possible that deleting a second gene will make the animal healthier? However, cell biological pathways often need to be tightly regulated, and this regulation usually incorporates positive and negative inputs to tune the output of the pathway. Thus, suppressor screens identify genes that provide reciprocal regulation to a biological process.

Acknowledgments

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Further Reading

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