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Neurogenetics of Neurotransmitter Release in *Caenorhabditis elegans*

RJ Hobson, KJ Yook, and EM Jorgensen, University of Utah, Salt Lake City, UT, USA

Glossary

d0005 **Endocytosis** The process of recycling lipids and proteins from the plasma membrane into internal vesicles.

d0010 **Exocytosis** The process where a cell directs the contents of a secretory vesicle to the extracellular space through the fusion of the secretory vesicle with the plasma membrane.

d0015 **Genetic screen** A procedure or test to identify individuals in a population with a phenotype of interest.

d0020 **Nematode** An unsegmented pseudocoelomate, also referred to as a roundworm. One of the most diverse animal species with over 28 000 described.

d0025 **Transgenic** Introduction of an exogenous piece of DNA (a transgene) into a living organism.

s0005 Using a Free-Living Nematode to Study Neurobiology

p0005 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 number and positions of the neurons are invariant between individuals (Figure 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 by 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 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, *C. elegans* can be propagated as a self-fertilizing hermaphrodite so it does not need a nervous system to search for mates and to reproduce. Males can be used to cross strains together to produce complex genotypes.

p0010 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 comprising hundreds of copies of the injected DNA. Multicopy transgenes can result in overexpression, dominant negative effects, toxicity, promoter titration, or even gene silencing. Some tissues, such as the germline or muscle, are refractory to expression of the 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 from use of the Mos transposon. The Mos1 transposon is a fly transposon that has 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 new technologies combined with traditional genetics of *C. elegans* allow for new approaches to the study the genetics of neurotransmission, for example, structure–function analyses of the proteins involved in exocytosis and endocytosis.

Genetic Dissection of Neurotransmission

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p0015 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 (Figure 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*.

Neurotransmitters

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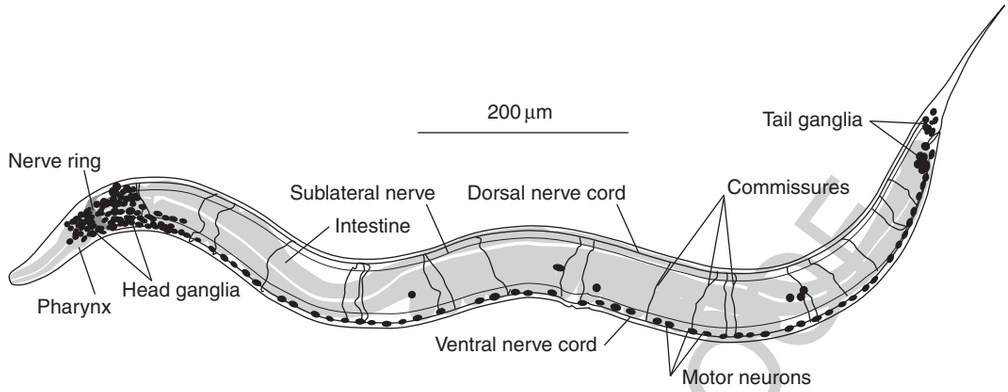
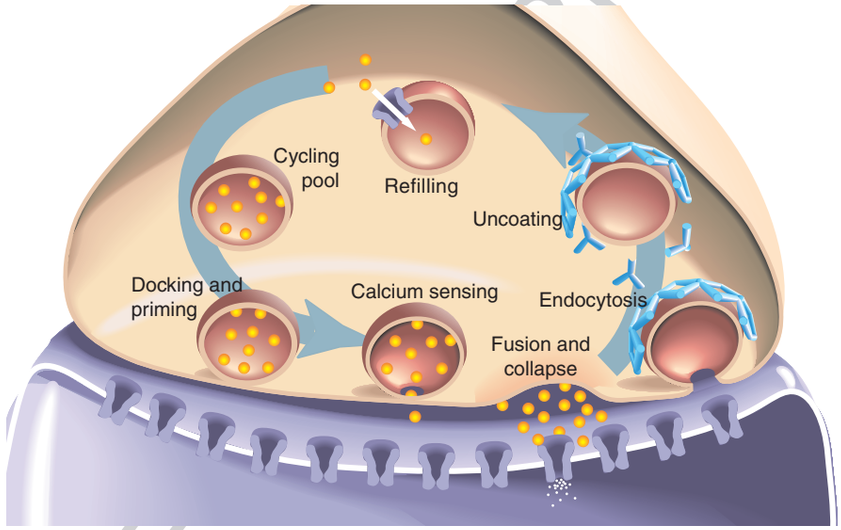
p0020 The original genetic screens for synaptic function involved the identification of mutant *C. elegans* that had difficulties moving. While these screens identified some of the genes involved in synaptic transmission, it was difficult to tease these apart from genes required for other nervous system functions, such as



f0005 **Figure 1** The adult nervous system of *C. elegans*. There are 302 neurons in an adult nematode. Most of the cell bodies of the neurons are found in the AU1 head ganglia, ventral nerve cord, or tail ganglia. Not all axon bundles are shown.

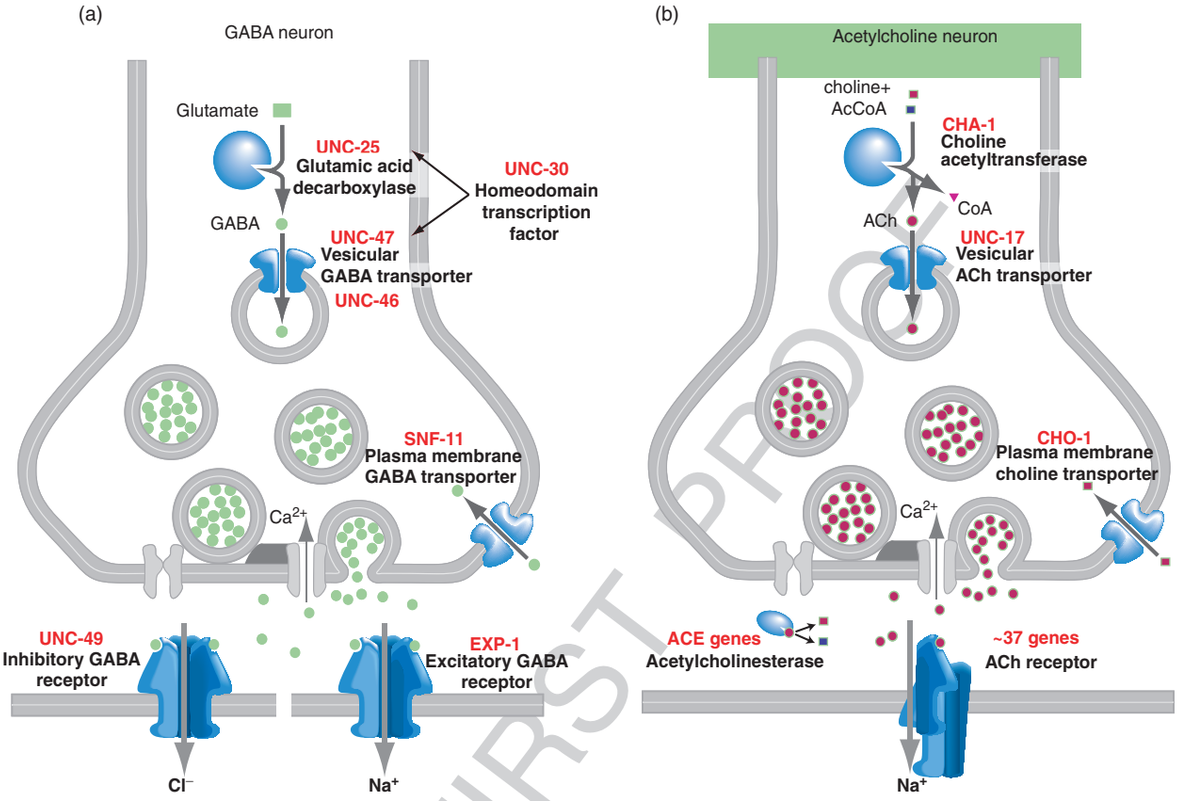


f0010 **Figure 2** The synaptic vesicle cycle. The presynaptic neuron releases neurotransmitter (yellow) from synaptic vesicles into the synaptic cleft. The vesicles dock and become primed for exocytosis. Calcium influx acts through a calcium sensor to stimulate fusion of the vesicle and plasma membranes. After fusion, membrane and vesicle proteins are recycled by endocytosis. Vesicles are refilled and re-enter the cycling pool.

development. To determine the molecules specifically involved in neurotransmitter release required the use of more focused AU4 screens. We will focus on the two best-studied neurotransmitters, gamma-aminobutyric acid (GABA) and acetylcholine, the two major neurotransmitters at the *C. elegans* neuromuscular junction.

p0025 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. Screens for shrinker mutants which mimicked the loss of GABA, identified five of the seven genes required for GABA function (**Figure 3**). These five 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 BAD-LAMP protein is required for localization of the GABA transporter, UNC-47. UNC-30 is a 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. The plasma membrane transporter *snf-11* was not identified in these screens, but rather was identified by molecular similarity. Mutation of this gene demonstrated that it is not required for GABA function. In addition, a novel excitatory GABA receptor *exp-1* was identified that is found in invertebrates but not in vertebrates.

p0030 Loss of acetylcholine neuron function leads to a lethal phenotype. However, it was still possible to identify weak alleles of the genes involved in acetylcholine transmission due to their resistance to acetylcholinesterase inhibitors. The most commonly used one 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 (**Figure 4**). Therefore, it is



f0015 **Figure 3** Neurotransmitter-specific molecules for GABA (a) and acetylcholine (b). Protein functions (black) and *C. elegans* three-letter protein names (red) are shown. GABA, gamma aminobutyric acid; Ach, Acetylcholine; AcCoA, Acetyl CoA.

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. While Aldicarb sensitivity is a measure of levels of acetylcholine release, it is sensitive to any mutation that affects the machinery of neurotransmitter release or synaptic development meaning that while it has been an extremely useful tool for understanding acetylcholine transmission, it has also been used to screen for proteins involved in general synaptic function.

p0035 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. However, at this time, molecular techniques were not advanced in *C. elegans* and the gene sequences could not be described. Two years later, acetylcholine receptors from the electric ray Torpedo were cloned in the laboratories of Numa, Heinemann, and Changeux. The genes encoding the acetylcholine receptors in *C. elegans* have now been identified from the genome of *C. elegans*. Ironically, the genome is particularly rich in acetylcholine subunits – there are 37 genes encoding different isoforms of acetylcholine receptors.

p0040 *C. elegans* has also provided some surprises in the diversity of neurotransmitters it uses. While the worm appears to have the full repertoire of classical transmitters and peptides a novel neurotransmitter regulates the defecation cycle. The cycle is a 50-s program comprising a posterior body contraction and expulsion of the contents of the gut. Contraction of the posterior body muscles is regulated by an intestomuscular 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 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 channels 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.

Trafficking of Vesicles

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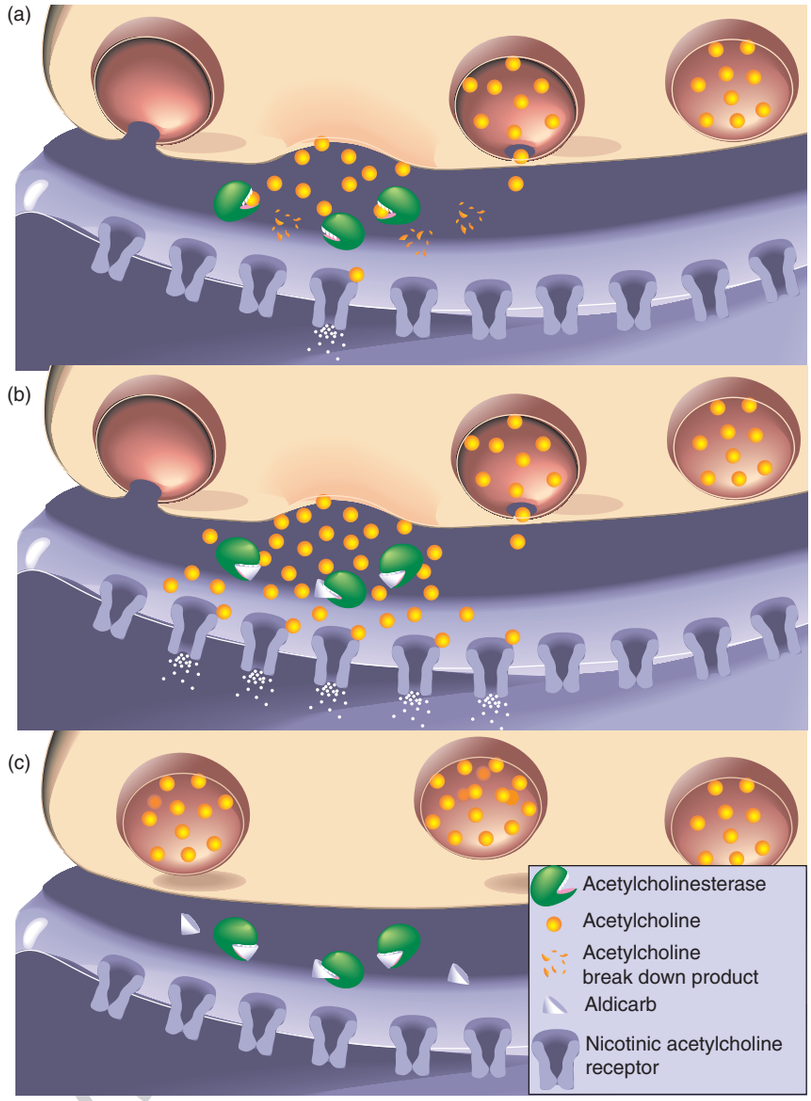
p0045 Proteins required for the functioning of all synapses include proteins required to transport materials from the cell body to the synapse and proteins required to dock, fuse, and recycle synaptic vesicles at the active zone. The cell body of the neuron is often far from the synapse. To transport 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.



f0020 **Figure 4** Aldicarb blocks acetylcholine degradation. (a) In a wild-type synapse, acetylcholine is rapidly degraded by acetylcholinesterase so that signaling at the synapse is rapid. (b) Aldicarb blocks the enzymatic cleft of acetylcholinesterase and acetylcholine accumulates in the cleft and leads to chronic activation 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. (c) In mutants with defects in exocytosis such as *unc-13*, the synapse releases little or no acetylcholine and the animal is resistant to Aldicarb.

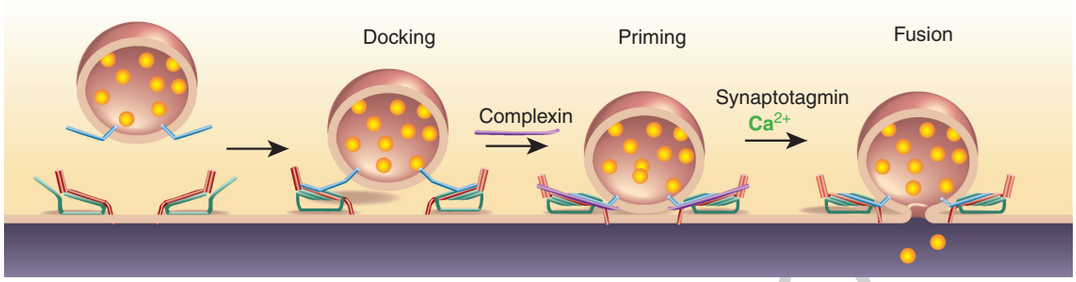
Vertebrate homologs have been discovered and comprise the Kinesin-3 family of kinesins.

s0025 Synaptic Vesicle Cycle

p0050 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 (Figure 2).

Exocytosis s0030

p0055 The fusion of synaptic vesicles with the plasma membrane requires the formation of the SNARE complex (Figure 5). The SNARE complex is comprised of three proteins: syntaxin/UNC-64, SNAP-25/RIC-4, 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 that 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 AU5



f0025 **Figure 5** Steps in exocytosis. Regulated fusion of the vesicle and plasma membranes requires several steps. First, the vesicle must dock with the plasma membrane. Docking requires the SNARE protein syntaxin (red). Although other docking components are not known, it is possible that docking involves the binding of the vesicular SNARE protein synaptobrevin (blue) with the plasma membrane SNARE proteins SNAP-25 (green) and syntaxin (red), possibly AU2 via the actions of UNC-18. 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. Upon calcium influx in the vesicle, synaptotagmin (not shown) senses the presence of calcium and stimulates fusion of the vesicle with the plasma membrane by the full winding of the SNARE proteins, resulting in the release of neurotransmitter into the synaptic cleft. The process of calcium sensing and vesicle fusion can occur as fast as 100 μs.

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 facilitory role in neurotransmission. UNC-18 has also been implicated in trafficking of syntaxin to the synapse. In animals with reduced UNC-13 function, neurotransmitter release is abolished. UNC-13 is required for the docking of vesicles at the active zone. UNC-13 is believed to convert the SNARE syntaxin from a closed configuration into an open configuration so that it can engage the other SNARE protein SNAP-25 and the vesicular SNARE protein synaptobrevin, and thereby facilitates priming of vesicles. Moreover, this protein is the target of modulatory cascades that increase 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 the association of UNC-13 with the plasma membrane and a concomitant increase in vesicle priming. Once the vesicle is primed the actual exocytosis fusion event is triggered by calcium influx. 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.

Endocytosis

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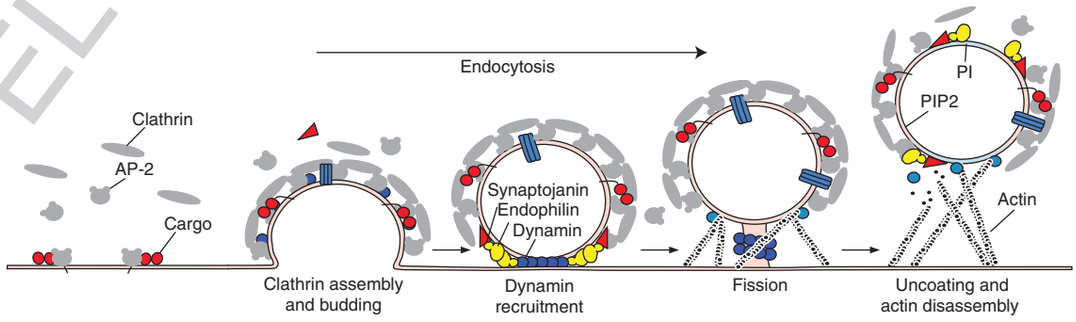
p0060 Once a vesicle fuses with the plasma membrane, the synaptic vesicle and its associated proteins are retrieved from the plasma membrane by endocytosis (Figure 6). Synaptic vesicle endocytosis is thought to be mediated by the formation of a clathrin cage which buds membrane into the cell. Surprisingly synaptotagmin/SNT-1 plays a dual role in the synaptic vesicle cycle, a phenotype first identified in *C. elegans* and subsequently identified in fly and mouse synaptotagmin 1 knockout neurons. In *snt-1* mutants, there is a striking loss of synaptic vesicles due to a failure in endocytosis. The C2 domains of synaptotagmin can bind the mu2 subunit of the AP2 clathrin adaptor complex and can also bind the mu2 homology domain of the endocytic adaptor protein stonin/UNC-41. One possible model is that synaptotagmin recruits clathrin adaptor proteins to the plasma membrane and they in turn initiate membrane endocytosis. Another gene, *unc-11*, which encodes an adaptor protein called AP180, recruits the synaptic vesicle protein synaptobrevin, in addition to clathrin, to the membrane targeted for endocytosis.



f0030 **Figure 6** Steps in endocytosis. After the fusion of synaptic vesicles, the vesicle constituents (lipids and proteins) are recycled from the plasma membrane. PI, phosatidylinositol; PIP_2, phosatidylinositol 4,5-bisphosphate.

Progress beyond the coated pit stage requires changes in the composition and shape of the membrane. One protein implicated in this process is synaptojanin/UNC-26 a polyphosphoinositide phosphatase which 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 large BAR domain protein endophilin/UNC-57 has nearly identical endocytic defects to synaptojanin/UNC-26. The BAR domain of endophilin is likely to bind and curve the membrane. When the clathrin coat is assembled around the invaginating vesicle, dynamin, encoded by *dyn-1*, cleaves the vesicle from the plasma membrane. 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 that bind PIP2 remain attached to synaptic vesicle lipids.

These studies assumed that clathrin played the most important role in synaptic vesicle endocytosis – it provides the scaffold that curves membranes. However, recent studies have suggested that clathrin may in fact not be essential for synaptic vesicle endocytosis. Studies using a temperature-sensitive allele of the clathrin heavy chain, revealed that although it is essential for receptor-mediated endocytosis in polarized epithelial cells, synaptic vesicles endocytosis appeared completely normal in the mutants. These results suggest that, at least in a resting synapse, clathrin is not essential for synaptic vesicle recycling. Furthermore, removal of stonin/UNC-41 or individual components of the AP2 complex has as severe a defect on synaptic vesicle number as synaptotagmin mutants, suggesting that the synaptotagmin is recruiting other components of the endocytic machinery, or that synaptotagmin plays an active role in endocytosis.

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 that 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.

Future

The *C. elegans* genome contains 20 043 genes and currently >90% have some form of nonsense mutation present in them. 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 new genes. However, further analyses suffer from two drawbacks common to all genetic studies: lethality and redundancy. To solve the issue of lethality, it is necessary to develop techniques that generate mosaic animals, that is, to remove a gene in a particular cell in a wild-type animal. Site-specific recombination, such as the FLP/FRT or Cre/Lox combinations, will be able to excise a gene when expressed in a specific cell. Although these enzymes have worked to activate a gene, they are not yet reliable enough to inactivate a gene reliably. Lethality can also work for the geneticist: suppressor screens can be used to identify mutations that restore viability. Such screens identify genes that provide reciprocal regulation to the same biological process.

Redundancy also creates difficulties for the geneticist. A number of loci exhibit genetic redundancy, that is, a phenotype is only observed when multiple genes are mutated. Enhancer screens can identify genes in these parallel pathways. In short, strains carrying mutations in known genes can be mutagenized to identify second-site mutations that exhibit synthetic phenotypes.

Further Reading

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GNT2 01032

Biographical Sketch

AU17 Robert J. Hobson

Karen J. Yook

Erik M. Jorgensen received his BSc degree in animal resources from the University of California at Berkeley in 1979. He received his PhD degree working with Dr. Richard Garber in the Department of Genetics at the University of Washington in 1989. Postdoctoral work was conducted in H. Robert Horvitz's laboratory at the Massachusetts Institute of Technology where Jorgensen initiated studies into the genetic basis of GABA transmission in the nematode *Caenorhabditis elegans*. In 1994, Jorgensen established his own laboratory in the Department of Biology at the University of Utah. In 2005, Jorgensen was named an Investigator of the Howard Hughes Medical Institute. Current studies in the laboratory focus on proteins required for both synaptic and nonsynaptic neurotransmission. In addition, the laboratory studies circuits such as those involved in sexual attraction or rhythmic behaviors. The laboratory is committed to the development of new techniques in genome manipulation and in fluorescence and electron microscopy.