

Neurogenetics of vesicular transporters in *C. elegans*

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ABSTRACT The nematode *Caenorhabditis elegans* has a number of advantages for the analysis of synaptic molecules. These include a simple nervous system in which all cells are identified and synaptic connectivity is known and reproducible, a large collection of mutants and powerful methods of genetic analysis, simple methods for the generation and analysis of transgenic animals, and a number of relatively simple quantifiable behaviors. Studies in *C. elegans* have made major contributions to our understanding of vesicular transmitter transporters. Two of the four classes of vesicular transporters so far identified (VACHT and VGAT) were first described and cloned in *C. elegans*; in both cases, the genes were first identified and cloned by means of mutations causing a suggestive phenotype (1, 2). The phenotypes of *eat-4* mutants and the cell biology of the EAT-4 protein were critical in the identification of this protein as the vesicular glutamate transporter (3, 4). In addition, the unusual gene structure associated with the cholinergic locus was first described in *C. elegans* (5). The biochemical properties of the nematode transporters are surprisingly similar to their vertebrate counterparts, and they can be assayed under similar conditions using the same types of mammalian cells (6, 7). In addition, mild and severe mutants (including knockouts) are available for each of the four *C. elegans* vesicular transporters, which has permitted a careful evaluation of the role(s) of vesicular transport in transmitter-specific behaviors. Accordingly, it seems appropriate at this time to present the current status of the field. In this review, we will first discuss the properties of *C. elegans* vesicular transporters and transporter mutants, and then explore some of the lessons and insights *C. elegans* research has provided to the field of vesicular transport.—Rand, J. B., Duerr, J. S., Frisby, D. L. Neurogenetics of vesicular transporters in *C. elegans*. *FASEB J.* 14, 2414–2422 (2000)

CAENORHABDITIS ELEGANS AS A MODEL ORGANISM

These studies rely on the strengths of *Caenorhabditis elegans* as a research organism: ease of manipulation, a simple nervous system, and powerful tools for the analysis of mutants and genes. Genetically, *C. elegans*

is advantageous because of its short generation time (3 days), its prolific progeny yield (280 per parent), its small size (1.5 mm long), and its ease of laboratory culture (on *Escherichia coli* lawns on agar in Petri dishes). There are now thousands of mutant strains of *C. elegans*, exhibiting a wide variety of behavioral, morphological, and developmental phenotypes, and hundreds of genes have now been mapped on the animals' six chromosomes.

C. elegans is particularly suited for studying neural function. Its nervous system has many morphological and biochemical similarities to those of mammals. Most proteins identified as important for neurotransmitter release have homologues in both *C. elegans* and mammals (8, 9). In addition, because *C. elegans* hermaphrodites can self-fertilize and produce progeny without mating, it is possible to maintain strains carrying severe neural defects that disrupt movement or other behaviors.

Cellularly, *C. elegans* is remarkably simple. At hatching, there are 550 somatic cells (10); over the course of the next 48 h, this number increases to produce 959 adult somatic cells (11). The adult contains 302 neurons, and reproducibility of neuron structure and connectivity has been demonstrated by serial section electron microscopy for most portions of the nervous system (12–15).

Molecular biology using this organism is simplified by its relatively small genome size of 10^8 bp, which has now been fully sequenced (16, 17). *C. elegans* can maintain injected DNA as extrachromosomal arrays, and it is therefore relatively straightforward to express cloned genes by transformation (18, 19).

NEUROTRANSMITTER STUDIES IN *C. ELEGANS*

Although the *C. elegans* nervous system has only 302 neurons, the diversity of classical neurotransmitters and neuropeptides seems comparable to that in vertebrate nervous systems (20). Analysis of specific neurotransmitters and their functions has relied on a

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combination of pharmacology, biochemistry, mutant analysis, and cell ablation studies. In particular, the relatively simple neural circuitry of *C. elegans* has made it possible to assess the involvement of specific neurotransmitters in particular behaviors.

CHOLINERGIC FUNCTION IN *C. ELEGANS*

Acetylcholine is the major excitatory neurotransmitter controlling motor functions in nematodes (21, 22). *C. elegans* contains the enzymes of acetylcholine synthesis and degradation (23–25), and acetylcholine is present in extracts (26–28). In addition, *C. elegans* are paralyzed by cholinesterase inhibitors (organophosphates and carbamates) and by nicotinic agonists (29).

The transmitter is synthesized by choline acetyltransferase (ChAT) (25), encoded by the *cha-1* gene (30). Partially ChAT-deficient mutants grow slowly and display deficits in a number of neuromuscular behaviors, such as locomotion, feeding, and defecation (30–33). ChAT-deficient mutants are also resistant to AChE inhibitors (30, 34), presumably because decreased synthesis of acetylcholine counteracts the toxin-induced accumulation of excess synaptic transmitter. Mutations that eliminate all gene function (null mutations) are lethal; mutant homozygotes are able to complete embryogenesis and hatch, but the young larvae are unable to move or feed normally, and they shrink and die within a few days (31).

Based on immunostaining with anti-ChAT antibodies, ~115 *C. elegans* neurons appear to be cholinergic; almost all of these cells are motor neurons (J. S. Duerr et al., unpublished results).

VACHT AND *unc-17* MUTANTS

Mutations in the *unc-17* gene (so named because mutations conferred *uncoordinated* locomotion) were first described by Brenner (29). In addition to the locomotion deficit, *unc-17* mutants with a slight amount of residual gene function are slow-growing, small, resistant to inhibitors of cholinesterase, and contain excess acetylcholine (26, 28, 29). Null mutations for *unc-17* are lethal, with the same phenotype displayed by null *cha-1* mutants (1, 31).

The *unc-17* gene was cloned and sequenced by Alfonso et al. (1); when it was first cloned, the *unc-17* gene product (UNC-17) had no homologs in the databases. The predicted UNC-17 protein contained 532 amino acid residues and 12 putative transmembrane domains. Immunocytochemical studies demonstrated that ChAT and UNC-17 were colocalized at the cellular and the subcellular level. Immunoreac-

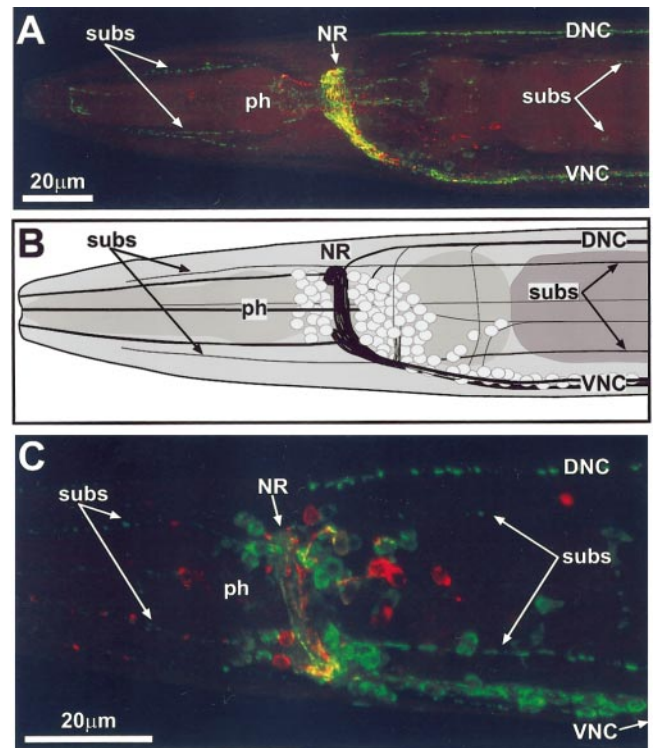


Figure 1. Distribution of immunoreactivity of VACHT (green) and VMAT (red) in *C. elegans*. *A*) VACHT and VMAT immunoreactivity in the head of a wild-type nematode. Immunoreactivity is seen in the nerve ring (NR), ventral nerve cord (VNC), dorsal nerve cord (DNC), and the dorsal and ventral sublateral nerve cords of the head and body (subs). In the pharynx (ph), numerous immunoreactive processes are also seen. Immunoreactivity is predominantly in synaptic regions along neuronal processes and is sparse in neuronal cell bodies (compare with diagram in panel *B*). The yellow regions in the ventral nerve cord are generally due to green (VACHT) and red (VMAT) positive regions that are very close, but are not overlapping. *B*) Diagram of the nervous system of the nematode illustrated in panel *A*. Neuronal processes are shown as black lines; neuronal somas are shown as white circles with gray outlines. The pharynx is indicated with medium gray; the neurons within the pharynx are not diagrammed for the sake of clarity. The beginning of the intestine is shown as a darker gray. *C*) The region around the nerve ring in an *unc-104* mutant at slightly higher magnification. In *unc-104* mutants, synaptic vesicles as well as VACHT and VMAT immunoreactivity are abnormally concentrated in cell bodies. The cell bodies of numerous neurons in the lateral ganglia and the ventral ganglion are apparent. Many neurons are immunoreactive for VACHT or VMAT, but very few are immunoreactive for both.

tivity to both proteins is present in synaptic regions of ~115 neurons, and additional experiments showed that UNC-17 was associated with synaptic vesicles (Fig. 1) (1). Almost all of the ChAT- and UNC-17-positive cells are (apparently excitatory) motor neurons. When the cloning of the rat VMATs was published (35, 36), it became clear that UNC-17 was related to the VMATs, and it was therefore likely to be the *C. elegans* VACHT (Fig. 2) (1). The *C. elegans* *unc-17* gene was then used to isolate vertebrate homologs, and the identity of the corresponding

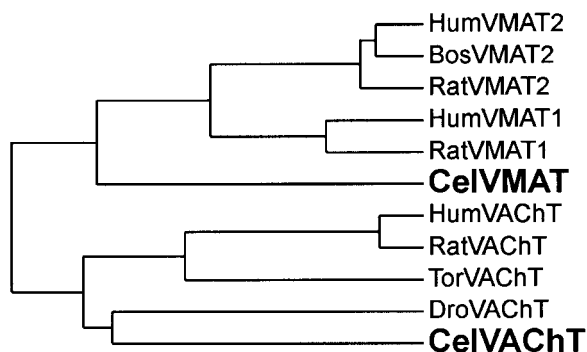


Figure 2. Dendrogram of published VMAT and VAcHT sequences. The PILEUP program (Genetics Computer Group Wisconsin Package, Version 8) was used. (Modified slightly from 7). Prefix abbreviations: Bos, bovine; Cel, *C. elegans*; Dro, *Drosophila*; Hum, human; Tor, *Torpedo*. The individual Genbank Accession numbers are: HumVMAT2, #L23205 (79); BosVMAT2, #U02876 (80); RatVMAT2, #L00603 (35); HumVMAT1, #U39905 (81); RatVMAT1, #M97380 (36); CelVMAT, (7); HumVAcHT, #U10554 (6); RatVAcHT, #U09211 (6); TorVAcHT #U05591 (37); DroVAcHT, #AF030197 (39); CelVAcHT, #L19621 (1).

gene products as VAcHTs was confirmed by binding and uptake studies (6, 37).

An unusual aspect of *unc-17* is its genomic organization: it is part of a complex transcription unit that also includes the *cha-1* gene (5). The *cha-1* and *unc-17* transcripts appear to be derived by alternative splicing of a common precursor. The two genes use a common 5'-untranslated exon; the remainder of the *unc-17* gene is nested within the long first intron of *cha-1* (Fig. 3). A similar genomic organization (i.e., the VAcHT gene nested within the first intron of the ChAT gene) was subsequently identified in mammals and in *Drosophila* (6, 38, 39). Thus, in mammals, insects, and nematodes, the synthesis and the vesicular transport of acetylcholine are coupled at the genomic level (Fig. 3). This suggests that the organization of this cholinergic locus is somehow important for its function. It is noteworthy that a similar organization is not found for VMAT or for VGAT.

CATECHOLAMINES IN *C. ELEGANS*

Three amine neurotransmitters have been identified thus far in *C. elegans*. Dopamine was originally identified in eight sensory neurons using the technique of formaldehyde-induced fluorescence (40). Exogenous dopamine inhibits locomotion and egg laying (41). Dopamine is synthesized in a two-step enzymatic process, using the enzymes tyrosine hydroxylase and aromatic amino acid decarboxylase (AAAD; often called DOPA decarboxylase). In *C. elegans*, these enzymes are encoded by the *cat-2* and *bas-1* genes, respectively (42, 43). Using mutations in these

genes, it has been possible to analyze the involvement of dopamine in specific behaviors. The deficits in such mutants include difficulties sensing and responding to the presence of food (42, 44). Males contain three additional pairs of dopamine-containing sensory neurons in the tail, and mutants lacking dopamine are deficient in male mating behaviors (42, 45).

Serotonin (5HT) has been identified in *C. elegans* neurons by formaldehyde-induced fluorescence (41) and by anti-5HT immunostaining (46, 47). In *C. elegans*, exogenous 5HT stimulates egg laying and pharyngeal pumping and inhibits locomotion and defecation (41, 48). There are 11 neurons in *C. elegans* with anti-5HT immunoreactivity (7, 42, 46); these include sensory neurons, interneurons, motor neurons, and secretory cells. Serotonin is synthesized by the sequential action of tryptophan hydroxylase (encoded by the *tph-1* gene) and AAAD (the same enzyme used for dopamine synthesis, encoded by *bas-1*) (42, 49). Mutant analysis and drug studies have shown that, in addition to regulating egg laying, serotonin mediates the response to starvation and is required for male mating behaviors (42, 44, 50, 51).

Epinephrine and norepinephrine were not detected in *C. elegans*; however, octopamine (*p*-hydroxyphenylethanolamine) was present in *C. elegans* homogenates, and exogenous octopamine inhibited egg laying and stimulated locomotion (41). In addition, the presence of N-acetyl serotonin has been reported in several parasitic nematodes, and tissue extracts of *Ascaris* and *C. elegans* contain an enzymatic

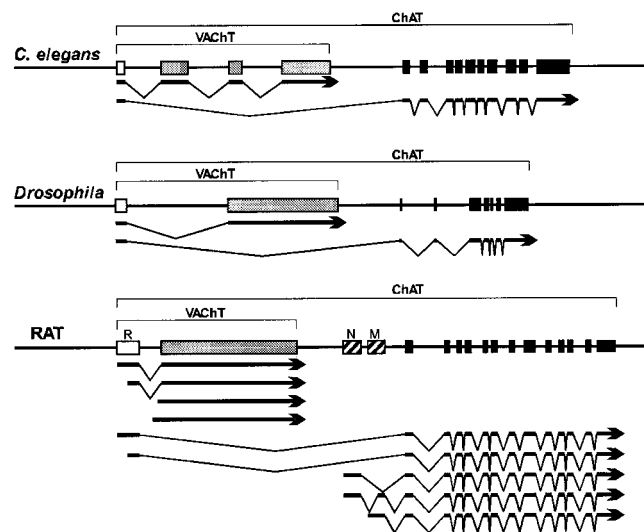


Figure 3. Organization of the cholinergic gene locus in *C. elegans*, *Drosophila*, and rat. The transcription and splicing patterns are shown for VAcHT and ChAT in the three species (5, 39, 78). VAcHT-specific exons are shown in gray, ChAT-specific exons in black, and shared exons in white. For the rat locus, the alternative N and M 5'-exons ChAT exons are shown with diagonal stripes. The three maps are not drawn to the same scale.

activity (arylalkyl amine N-acetyl transferase) capable of acetylating serotonin, dopamine, and octopamine (52, 53). It is not known whether the N-acetylated derivatives are merely catabolic intermediates or if they might represent additional or alternative neurotransmitters.

VMAT AND *cat-1* MUTANTS

The *C. elegans* Genome Sequencing Consortium identified sequence on genomic cosmid W01C8 that encoded a VMAT-like protein; this appears to be the only VMAT homologue in the *C. elegans* genome. Analysis of cDNAs indicated an open reading frame corresponding to a 553 amino acid protein; the predicted *C. elegans* protein is 47% identical to rat VMAT1 and 49% identical to rat VMAT2 (Fig. 2) (7). When expressed in mammalian cells, the *C. elegans* protein is functional and mediates time-dependent transport of serotonin and dopamine; transport is reserpine sensitive and tetrabenazine sensitive, and is competitively inhibited by dopamine, octopamine, tyramine, norepinephrine, and histamine (7).

Immunolocalization studies indicate that the VMAT protein is primarily localized to synaptic regions of a subset of neurons and is associated with synaptic vesicles (Fig. 1) (7). There are 25 VMAT-positive neurons; these include the 8 dopamine-containing cells and the 11 serotonin-containing neurons, as well as a few still unidentified cells. These neurons are (with at least two clear exceptions) distinct from those that are immunopositive for VACHT.

The previously identified *cat-1* gene corresponds to the *C. elegans* VMAT homologue. In *cat-1* mutants, levels of dopamine (visualized by formaldehyde-induced fluorescence; 40) and 5HT (visualized by immunocytochemistry; 42) are decreased in neuronal processes and increased in cell bodies. Furthermore, the apparent abundance of dopamine is decreased by ~60%, and VMAT immunoreactivity is eliminated in *cat-1* mutants (7, 40). It is noteworthy that these are the same phenotypes obtained by treating wild-type animals with reserpine (40). *cat-1* mutants are also deficient in dopamine- and 5HT-mediated behaviors, such as slowing their rate of locomotion in the presence of food, egg laying, and male mating (7, 42).

GABA FUNCTION IN *C. ELEGANS*

The evidence for GABA function in *C. elegans* includes pharmacological studies using GABA-related compounds, immunohistochemical demonstration of the presence of GABA in specific cells, and

analysis of mutants defective in GABA synthesis and function (54, 55). Twenty-six neurons contain GABA immunoreactivity; most of these are inhibitory motor neurons, but some are apparently excitatory GABAergic motor neurons (55). In each of these cells, the GABA immunoreactivity is uniformly distributed throughout the cytoplasm and is not restricted to synaptic regions (55).

Mutants with GABAergic transmission defects are viable, although they have several motor defects. The most obvious phenotype is a tendency to contract dorsal and ventral body wall muscle simultaneously in response to touch ('shrinker' phenotype); this appears to result from lack of function of the GABA-containing DD and VD inhibitory motor neurons (54, 55).

GABA is synthesized from glutamic acid by the enzyme glutamic acid decarboxylase (GAD). In *C. elegans*, *unc-25* encodes a protein that is ~45% identical to mammalian GAD (56). *unc-25* mutants have no apparent GABA (54) and extracts prepared from these animals lack GAD activity (C. Johnson and A. Stretton, personal communication). The *unc-25* GAD gene is expressed in 26 neurons (56), which correspond to the 26 GABA-containing neurons.

VGAT AND *unc-47* MUTANTS

Mutants of the *unc-47* gene were first reported by Brenner (29) and extensively characterized by McIntire et al. (54). These mutants were deficient in GABA-mediated behaviors and accumulated excess GABA in presynaptic neurons, but displayed normal responses to GABA agonists. This led to the suggestion that *unc-47* might encode a VGAT (54). Subsequent cloning of the locus revealed a 486 amino acid protein containing 10 putative transmembrane domains, bearing a weak similarity to some plant amino acid permeases (2). The UNC-47 protein was associated with synaptic vesicles and its expression was limited to the 26 previously identified GABA-containing neurons (2).

A rat homologue of the UNC-47 protein (38% identical) conferred GABA transport activity to PC12 cells, with an apparent K_m of ~5 mM (2). Additional studies have suggested that the mammalian transporter is also capable of vesicular transport of glycine (57); it would therefore seem more appropriate to consider the protein to be a vesicular inhibitory amino acid transporter (VIAAT) (58, 59).

GLUTAMATE FUNCTION IN *C. ELEGANS*

Molecular studies have identified and characterized several of the components of glutamatergic transmis-

sion in *C. elegans*, and mutant analysis has suggested a role for glutamate in specific neural circuits and the behaviors they mediate.

A *C. elegans* plasma membrane glutamate transporter (*glt-1*) similar to the vertebrate excitatory amino acid transporters has been cloned and shown to exhibit glutamate transport activity when expressed in *Xenopus* oocytes (60–62). In addition, several genes encoding glutamate receptor subunits have been cloned and studied; these include *glr-1*, which encodes an AMPA-type receptor (63, 64), and three genes—*avr-14*, *avr-15*, and *glc-1*—that encode subunits of glutamate-gated chloride channels (65–68). These glutamate-gated chloride channels have been shown to be the sites of action of the antiparasitic drug ivermectin (69).

In general, the identification of glutamate releasing neurons and glutamate-dependent behaviors has been inferred from analysis of the phenotypes of *glr-1* mutants (and *eat-4* mutants, described below) and from the expression patterns of the receptor proteins. These studies suggest that glutamate is released by many sensory neurons and interneurons, and a few of the behavioral circuits have been identified. These include 1) the response to light mechanical stimulation of the head mediated by the ASH sensory neurons through the GLR-1 postsynaptic receptor (63, 64); 2) the response to strong mechanical stimulation in the anterior body mediated by the ALM and AVM sensory neurons through the AVR-15 postsynaptic receptor (3); and 3) the effects of the M3 motor neuron on the pharyngeal muscle, mediated through the AVR-15 postsynaptic receptor (described below).

VGlut AND *eat-4* MUTANTS

Mutations in the *eat-4* gene were originally identified because of altered feeding behavior (33). The M3 cells are inhibitory motor neurons that trigger repolarization of the pharyngeal muscle, and many of the pharyngeal pumping defects present in *eat-4* mutants are phenocopied by laser ablation of the M3 neurons (70). M3 neurotransmission is mediated by the postsynaptic *avr-15* glutamate receptor (67); after M3 ablation, the function(s) of the M3 neurons could be restored by application of glutamate but not by other putative neurotransmitters (71). This strongly suggested that glutamate was the neurotransmitter released by the M3 cells. In addition, because the pharyngeal muscles of *eat-4* mutants have normal postsynaptic responses to glutamate, the *eat-4* gene product EAT-4 apparently functions in the presynaptic (M3) neurons (67).

In addition to the pharyngeal effects, *eat-4* mutants exhibit many of the sensory defects associated with

glr-1 mutants (3). There are also alterations in the habituation behavior of *eat-4* animals: they habituate more rapidly than wild-type, recover from habituation more slowly, and show no dishabituation (72).

Cloning and sequencing of the *eat-4* gene showed that the EAT-4 protein was quite similar to the previously identified mammalian brain-specific, sodium-dependent inorganic phosphate transporter BNPI (3, 73). The EAT-4 protein is expressed in at least 38 neurons; these include the M3 cells as well as a number of sensory neurons known to be presynaptic to GLR-1-expressing interneurons (3).

Although the mammalian BNPI protein was originally characterized as a plasma membrane phosphate transporter, reexamination of its properties showed that it was associated preferentially with small synaptic vesicles, rather than with the plasma membrane (74), and that the expressed protein could transport glutamate (4). These data, together with the EAT-4 expression pattern and the *eat-4* mutant phenotypes, suggested strongly that BNPI and EAT-4 were in fact vesicular glutamate transporters.

USE OF REPORTERS AND STUDIES OF GENE REGULATION

One of the advantages of simple model organisms (e.g., *C. elegans* and *Drosophila*) is the relative ease with which genes and altered gene constructs may be introduced and expressed. This has permitted the use of reporter proteins to monitor the patterns of gene expression. A further advantage of *C. elegans* is its transparency; in combination with the new generation of fluorescent reporter proteins (e.g., GFP), it is now possible to monitor cellular expression patterns directly, in live, developing nematodes (75, 76).

For example, in a transgenic *C. elegans* strain containing a GFP reporter gene under the control of the *unc-17-cha-1* promoter region, strong GFP expression is seen in cholinergic neurons and not in other cells (Fig. 4). Similarly, GFP constructs have been used to demonstrate that both *unc-25* and *unc-47* are positively regulated by the UNC-30 homeodomain-containing transcription factor (77). It is noteworthy that even though *unc-25* and *unc-47* are both expressed in all 26 of the GABA-positive cells, their regulation by UNC-30 occurs in only 19 of these cells. These are the 19 so-called type D Inhibitory motor neurons, which are responsible for the reciprocal (ventral-dorsal) inhibition of body muscle contraction involved in sinusoidal locomotion (55).

Because of the relative ease with which reporter genes can be engineered and expressed, it is possible to perform relatively sophisticated promoter analysis

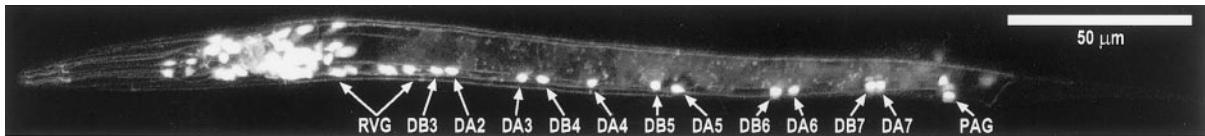


Figure 4. A VACHT reporter gene identifies cholinergic neurons in *C. elegans*. A 3.2 kb genomic fragment (extending upstream from the first *unc-17* exon to a *SnaI* restriction site) was cloned into the first multiple cloning site of a *C. elegans* expression plasmid containing a GFP reporter gene (19). The genomic fragment contains the necessary information for correct cellular expression of *unc-17* and *cha-1* (Frisby et al., unpublished results). The GFP reporter contains a nuclear localization signal. The plasmid was transformed into *C. elegans* using standard methods (19), except that a plasmid containing the wild-type *pha-1* cDNA (gift of Heinke and Ralf Schnabel, Max-Planck-Institute für Biochemie, Martinsried) was used as a transformation marker (7). Shown is a first-stage (L1) transgenic animal; anterior is to the left and ventral is down. The nuclear-localized GFP is expressed in 2 of the 3 classes of motor neurons in the L1 ventral nerve cord: the 7 DB neurons and the 9 DA neurons (13). DA8 and DA9 are both located in the pre-anal ganglion (PAG), and the DA1, DB1, and DB2 cell bodies are in the retrovesicular ganglion (RVG).

of *C. elegans* genes. However, there are significant experimental differences between promoter analysis in *C. elegans* and mammals. In both cases, a common experimental design involves ‘promoter bashing’ using ordered deletions of regulatory regions, followed by analysis of reporter expression. In *C. elegans* (or *Drosophila*), the expression of the reporter gene is observed in living animals, with very little control over the humoral environment of each differentiating neuron. Mammalian regulation experiments generally involve transfected cell lines and often measure the response of the reporter to a particular signal, e.g., NGF. In this case, there is great experimental control over the humoral environment of the cells, but little or no positional information available to let a cell know where it is in the organism. As a result, promoter analysis in worms or flies will tell you that a particular element is required for gene expression in a specific set of cells, whereas promoter analysis in mammals will tell you that a particular element is involved in the response to NGF. This is not to say that the genes are regulated differently in the two taxa (although they may be), but rather that the type of information we can readily obtain about the regulation is different.

LESSONS AND INSIGHTS FROM *C. ELEGANS*

In addition to the initial identification of VACHT and VGAT (and a major role in identification of VGluT), *C. elegans* research has made important contributions to our understanding of the biology of vesicular neurotransmitter transport. The biological information derives in part from the simplicity of the nematode body plan, particularly its nervous system. Often a neurotransmitter is used by only a limited number of neurons and is required for a (relatively) limited number of nonvital functions. For many transmitters, the biosynthetic enzymes are known and cloned, and mutants are available that are deficient in the synthesis of each. It is therefore possible to compare specific behaviors in synthesis-

deficient mutants and transport-deficient mutants or to compare either type of genetic lesion with laser ablation of the transmitter-containing cells.

Evolution of VMAT isoforms: *C. elegans*

Although clearly a close structural relative of the mammalian VMATs, *C. elegans* VMAT is neither a VMAT1 nor a VMAT2 (Fig. 2). Since there is only a single VMAT gene in the *C. elegans* genome, it appears that the two mammalian VMAT genes diverged from each other subsequent to the divergence of nematode and mammalian ancestors. However, the transport properties of *C. elegans* VMAT (in particular, its affinity for histamine) appear more like those of the mammalian VMAT2 (‘neuronal’) isoform than the VMAT1 (‘neuroendocrine’) isoform (7). The ability of *C. elegans* VMAT to recognize histamine suggests that the ability to transport histamine was not a function acquired by VMAT2, but rather a preexisting trait of VMAT that was retained by VMAT2 and lost by VMAT1.

How essential is vesicular transport?

As described above, *cha-1* (ACh synthesis) and *unc-17* (ACh vesicular transport) mutants have the same phenotypes, and *unc-25* (GABA synthesis) and *unc-47* (GABA vesicular transport) mutants have the same phenotypes. It therefore appears that for these two neurotransmitters, loss of vesicular transport seems to be functionally equivalent to loss of transmitter synthesis and that vesicular transport is therefore essential for neural function. This is not meant to exclude the possibility of nonvesicular release mechanisms for these transmitters (e.g., GABA efflux through plasma membrane GABA transporters); however, under standard laboratory conditions, nonvesicular release makes little or no contribution to the development of the nervous system or the behavior of the animals.

For some neurotransmitters and for some cells, loss of vesicular transport is equivalent to loss of cell

function. Thus, for example, the dopamine-related phenotypes associated with loss of VMAT (*cat-1* mutants) are quite similar to those associated with ablation of the 8 dopaminergic sensory neurons, and the phenotypes associated with loss of VGAT (*unc-47* mutants) are quite similar to those associated with ablation of the GABAergic motor neurons (7, 44, 54, 55).

In other cases, however, loss of vesicular transport is associated with only a partial loss of cell function. For example, although the HSN cells (which regulate egg laying) contain both serotonin and VMAT, mutational elimination of either has a much milder effect than laser ablation of the HSN cells (7, 42, 46). Discrepancies such as these point to the involvement of other neurotransmitters and/or neuropeptides in the functioning of these cells (51). Another example is the difference between the loss of VGluT (EAT-4) expression in the ALM and AVM sensory neurons and ablations of these cells; this is presumably because the ALM and AVM cells make electrical as well as chemical synapses onto their postsynaptic partners, and in *eat-4* mutants only the chemical transmission is compromised (3).

Regulation of cytoplasmic transmitter levels

In vesicular transport mutants, it is possible to unmask some of the homeostatic mechanisms involved in the regulation of transmitter levels. A striking difference between the acetylcholine and GABA vesicular transporters on the one hand, and the vesicular monoamine transporter on the other is that for both VAcHT and VGAT mutants, there is a net accumulation of the transmitter (presumably in the presynaptic cytoplasm) (26, 28, 55), whereas VMAT mutants are associated with a decrease in presynaptic transmitter (7, 40). Presumably, sequestering the dopamine or serotonin into synaptic vesicles leads to a net increase in the total levels of dopamine and serotonin, but it also seems that the biogenic amines in the cytoplasmic compartment are maintained at a low level whether or not there is sequestration. There are several plausible explanations for this. Sequestration might improve the stability of the amines, either because of the low pH or by protection from catabolic enzyme activities. In addition, it is likely that *C. elegans* tyrosine hydroxylase is similar to its vertebrate cousins and its activity is feedback-inhibited by dopamine.

Regulation of the cholinergic gene locus

Although it has long seemed likely that the expression of transmitter-specific genes would be coregulated, the genomic organization of the ChAT and VAcHT genes provided a plausible mechanism for

such regulation. In theory, if the mRNAs for both ChAT and VAcHT were derived from the same primary transcript, one would not expect transcriptional initiation factors to have any differential effects on ChAT and VAcHT expression. In mammals, however, there are several documented ChAT-specific and VAcHT-specific promoters, and transcripts derived from these promoters appear to be more abundant than transcripts derived by alternative splicing from the common (R-exon) promoter (reviewed in 78). It is therefore likely that ChAT and VAcHT would be differentially expressed in some cells and/or at some developmental stages.

Immunostaining studies in *C. elegans* indicate that ChAT and VAcHT proteins are present in the same cells at the same times (1; Duerr et al., unpublished results), although it would be difficult to detect subtle changes in the relative expression of the two proteins or differential expression limited to a few cells and/or brief developmental stages. Thus far, we have been unable to demonstrate the existence of additional (ChAT-specific or VAcHT-specific) promoters in *C. elegans* (unpublished observations); it therefore seems likely that there is only a single type of primary transcript, which is then processed to yield either a *cha-1* mRNA or an *unc-17* mRNA. Even under these conditions, it is possible that regulation of the alternative splicing (or other post-transcriptional mechanisms) or translation control mechanisms could lead to differential ChAT and VAcHT expression. **[F]**

Note added in proof: In addition to Bellocchio et al. cited in the text (4), another recent paper identifies BNPI as the vesicular glutamate transporter [Takamori, S., Rhee, J. S., Rosenmund, C., and Jahn, R. (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature (London)* **407**, 189–194]

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