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Identification of Major Classes of Cholinergic Neurons in the Nematode *Caenorhabditis elegans*

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ABSTRACT

The neurotransmitter acetylcholine (ACh) is specifically synthesized by the enzyme choline acetyltransferase (ChAT). Subsequently, it is loaded into synaptic vesicles by a specific vesicular acetylcholine transporter (VAChT). We have generated antibodies that recognize ChAT or VAChT in a model organism, the nematode *Caenorhabditis elegans*, in order to examine the subcellular and cellular distributions of these cholinergic proteins. ChAT and VAChT are found in the same neurons, including more than one-third of the 302 total neurons present in the adult hermaphrodite. VAChT is found in synaptic regions, whereas ChAT appears to exist in two forms in neurons, a synapse-enriched form and a more evenly distributed possibly cytosolic form. We have used antibodies to identify the cholinergic neurons in the body of larval and adult hermaphrodites. All of the classes of putative excitatory motor neurons in the ventral nerve cord appear to be cholinergic: the DA and DB neurons in the first larval stage and the AS, DA, DB, VA, VB, and VC neurons in the adult. In addition, several interneurons with somas in the tail and processes in the tail or body are cholinergic; sensory neurons are generally not cholinergic. Description of the normal pattern of cholinergic proteins and neurons will improve our understanding of the role of cholinergic neurons in the behavior and development of this model organism. J. Comp. Neurol. 506:398–408, 2008.

Indexing terms: acetylcholine; ChAT; VAChT; *C. elegans*; choline acetyltransferase; vesicular acetylcholine transporter

The nematode *Caenorhabditis elegans* is well suited for studies examining the development and function of the cholinergic nervous system. *C. elegans* has a simple and invariant anatomy; the adult hermaphrodite has 302 neurons whose connectivity has been described (summarized in WormAtlas, 2007). The *C. elegans* genome is well described (WormBase, 2007), and many molecular tools are available (for review see WormBook, 2007). Genetic, molecular, and physiological techniques are being used to determine the involvement of particular neurons in specific behaviors (for review see WormBook, 2007). The identification of cholinergic markers in *C. elegans* (Alfonso et al., 1993, 1994a) allows us to use the strengths of this system to develop a complete wiring diagram for a cholinergic nervous system.

Electrophysiological, biochemical, and genetic experiments suggest that acetylcholine (ACh) is a major excitatory neurotransmitter at neuromuscular junctions in nematodes. Decades ago, electrophysiological responses to ACh in muscles of the parasitic nematode *Ascaris suum* were demonstrated (del Castillo et al., 1963); more recently, *C. elegans* muscles and neurons have also been

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shown to respond to ACh (for review see Francis et al., 2003). ACh is synthesized by the enzyme choline acetyltransferase (ChAT); ChAT activity has been demonstrated in excitatory motor neurons in *A. suum* (Johnson and Stretton, 1985). In *C. elegans*, ChAT is encoded by the gene *cha-1*; mutants show severe changes in locomotion, pharyngeal pumping, egg laying, and other behaviors (Rand and Russell, 1984; Alfonso et al., 1994a). If ChAT activity is totally lacking, embryos hatch but are almost paralyzed and slowly shrink and die (Rand, 1989). The response to ACh is mediated via multiple types of ionotropic nicotinic ACh receptors (for review see Brown et al., 2006), G-protein-linked muscarinic receptors (Lee et al., 1999, 2000; Min et al., 2000), and ACh-gated chloride channels (Putrenko et al., 2005); receptors are present on muscles and neurons (for review see Francis et al., 2003).

Because ACh itself cannot be readily localized by histochemical or immunological techniques, antibodies to ChAT have been used to identify putative cholinergic neurons. Antibodies to the vesicular acetylcholine transporter (VAChT), which loads ACh into synaptic vesicles, also appear to be specific to cholinergic neurons; VAChT and ChAT are coexpressed in neurons in *C. elegans* and mammals (Alfonso et al., 1993; Weihe et al., 1996; Gilmor et al., 1996).

In *C. elegans*, ChAT and VAChT are encoded by the *cha-1* and *unc-17* genes, respectively (Alfonso et al., 1993, 1994b). We now report the production and use of antibodies to *C. elegans* ChAT and VAChT. ChAT appears to be present in two forms, one fairly uniformly distributed and one that is enriched in synaptic regions; VAChT is primarily synaptic. In the adult ventral nerve cord (VNC), six classes of motor neurons, three innervating dorsal body muscles and three innervating ventral body muscles, are cholinergic; these complement the two described γ-aminobutyric acid (GABA)-ergic classes (McIntire et al., 1993). In addition, several other motor neurons and interneurons in the body and the tail appear to be cholinergic.

MATERIALS AND METHODS

Growth and strains

Nematodes were grown on *E. coli* grown on NGM agar (Stiernagle, 2006). *C. elegans* strains used included wild-type Bristol strain N2 (Brenner, 1974) and strains with the following mutations: *cha-1(md39ts)*, *unc-17(e245)*, *ctIs43* [*Pdbl-1::GFP, unc-119(+)*]; dbl-1::GFP; unc-119(+)] (Suzuki et al., 1999), *inIs179* [*Pida-1::GFP*] (Zahn et al., 2001), *mgIs21* [*Plin-11-ABCDE::GFP; rol-6*] (Hobert et al., 1998); *otIs107* [*ser-2promoter1::GFP; lin-15(+)*] (Tsalik et al., 2003), and *oxIs12* [*Punc-47::GFP; lin-15(+)*] (McIntire et al., 1997). The *cha-1(md39ts)* mutation is a temperature-sensitive allele that is viable and slightly uncoordinated at 15°C and sterile and virtually paralyzed at 25°C; it was isolated in a noncomplementation screen as previously described (Alfonso et al., 1993). RM980, an *unc-104(e1265)* strain containing an uncharacterized partial suppressor, was crossed to strains containing green fluorescent protein (GFP) expressed in specific neurons to generate mutants in which GFP was expressed in an *unc-104* background. Most of these *unc-104*;GFP strains were significantly less healthy than *unc-104* alone. Several of the *C. elegans* strains as well as *Caenorhabditis briggsae* (AF16), *Caenorhabditis remanei* (EM464), and a *Pellioditis* sp. (DF5039) were provided by the Caenorhabditis Genetics Center (St. Paul, MN). Unless otherwise noted, references to wild-type nematodes refer to *C. elegans*.

Synthesis of fusion proteins

Fusion proteins were constructed using maltose binding protein (MBP) vectors (pMAL-c or pIH902; New England BioLabs, Beverly, MA). The ChAT-MBP fusion vector was synthesized by inserting a *cha-1* cDNA containing the complete open reading frame (approximately 1.9 kb) for ChAT (Alfonso et al., 1994a) into the StuI site. The VAChT-MBP fusion vector was synthesized by inserting a full-length cDNA for *unc-17* (Alfonso et al., 1993) between the StuI and PstI sites. *E. coli* (DH5α) were transfected and induced to synthesize fusion protein; fusion proteins were purified from bacterial extracts by passage over amylose columns. The ChAT-MBP protein was cleaved by incubation with factor Xa to generate antigen. VAChT-MBP could not be stably cleaved by factor Xa under a variety of conditions. Therefore, VAChT-MBP was frozen after purification and the intact fusion protein was used as an antigen.

Generation of Antibodies to ChAT and VAChT

Initially, four *C. elegans* ChAT and four *C. elegans* VAChT synthetic peptides were used as immunogens in 30 mice, 10 rabbits, and 25 chickens. Synthetic peptides were left free or were cross-linked with glutaraldehyde to serum albumin (Duerr, 2006). Immunizations with two cross-linked peptides were successful; serum from a rabbit (R388) temporarily bound to the albumin-coupled ChAT peptide AFRSDPRTDLQHFK, and serum from another rabbit (R383) bound to the albumin-coupled VAChT peptide NPHRRGTDSHGEKVQGT. Fusion proteins were used alone or in addition to cross-linked peptides as immunogens in additional animals (10 mice, 3 rabbits, and 6 chickens). None of the polyclonal sera generated consistently recognized ChAT in fixed tissue. One chicken (C49) temporarily produced antibodies to ChAT that exhibited a weak staining pattern similar to that seen with R388 anti-ChAT. One of three fusions of spleens from mice immunized with ChAT fusion protein and peptides yielded five mouse monoclonal antibodies (MAbs 1401, 1402, 1414, 1415, and 1432) that recognize ChAT fusion protein but do not bind to any individual albumin-coupled synthetic peptide. Antibodies that recognize VAChT were generated in three chickens (C94, C95, and C96), and one of two fusions produced a mouse monoclonal antibody that recognizes VAChT fusion protein (MAb1403).

Purification of Antibodies to ChAT and VAChT

Supernatants were used or mouse antibodies were purified from ascites with an ImmunoPure Plus Kit (Pierce, Rockford, IL). Chicken IgY antibodies were isolated from egg yolks with sequential PEG precipitations and chicken antibody preparations and rabbit sera were affinity purified. Occasionally, antibodies generated against ChAT or VAChT were affinity depleted before affinity purification as follows. Dilute serum was preincubated with a nitrocellulose membrane containing a different fusion protein (e.g., anti-VAChT-MBP sera was incubated with a ChAT-

MBP membrane) or maltose binding protein alone before affinity purification (Duerr, 2006).

Western transfers

Fusion proteins purified from *Escherichia coli* were separated, transferred to nitrocellulose membranes (Sartorius, Goettingen, Germany), and incubated as previously described (Duerr, 2006). Bound antibody was visualized after 5–15 minutes of incubation in TMB or LumiGlo chemiluminescent substrate (Kirkegaard and Perry Laboratories, Gaithersberg, MD). For Western transfers of endogenous protein, mixed populations of *C. elegans* were rinsed, incubated in solubilization buffer with protease inhibitors, microwaved, and diluted in protein sample buffer. The mixture was boiled for 7 minutes (for anti-ChAT) or incubated at 70°C for 20 minutes (for anti-VAChT), passed through a 26-gauge needle, spun for 1 minute at high speed, then immediately frozen or run out on a gel for Western analysis.

Immunocytochemistry

Immunocytochemistry was performed as previously described, using methanol acetone fixation and primary antibodies to ChAT or VAChT from diluted cell supernatants or from purified polyclonal serum at 1:50–1:200 (Duerr, 2006). Rabbit antisera against GFP were obtained from Invitrogen (No. A-6455, raised against GFP purified directly from the jellyfish *Aequorea victoria*) and from Clontech (No. 632377, raised against a mixture of peptides from the middle and C-terminus of *A. victoria* GFP). We have established specificity for both types of GFP antibodies: in each case, staining is seen only in animals expressing a GFP transgene. In addition, for several transgenic strains, we find equivalent immunostaining using the two different anti-GFP antibodies. Rabbit antisera against the UNC-86 transcription factor (prepared from *E. coli*-expressed UNC-86 protein) were obtained from J.Y. Sze (Albert Einstein College of Medicine). With indirect immunofluorescence, affinity-purified UNC-86 antibodies stain 47 cell nuclei in wild-type animals, but an *unc-86* null mutant shows no specific staining (Finney and Ruvkun, 1990); these antibodies therefore appear to be specific for the UNC-86 protein. Stained nematodes were examined with a Zeiss Axiophot microscope.

Transgenic animals

Cosmid F57G7 (a gift of Alan Coulson, Wellcome Trust Sanger Institute) contains the entire coding sequences for *cha-1* and *unc-17* as well as 13 kb upstream of the first exon and 11 kb downstream of the *cha-1* poly-A addition site (Alfonso et al., 1994a). F57G7 (concentration 15 μg/ml) was injected with or without a plasmid containing the dominant marker *rol-6(su1006)* (220 μg/ml) into *unc-17(e245)* or *cha-1(md39ts)* mutants using standard *C. elegans* transformation techniques (Mello and Fire, 1995). F_1 transformants were identified by their improved growth and coordination and, in some cases, roller phenotype and were used to establish six independent transformed lines for each condition.

Image acquisition and processing

Confocal images were taken with a Leica TCS NT or a Zeiss LSM-510 scanning confocal microscope. Confocal collection parameters were adjusted to allow full utilization of a 256-level gray scale for each fluorochrome. Files were saved with Zeiss or Leica software as single image planes or as maximum projections of image series, then exported as TIFF files. In Adobe Photoshop, the false colors were set and brightness and contrast (gamma value and range) were adjusted. In some cases, the background outside of the nematode of interest was set as black (e.g., in Fig. 1, a portion of another nematode was "removed" by setting the background to black). No other image manipulations were performed. Image enhancement of other images was similarly done in Adobe Photoshop, adjusting brightness and contrast (gamma value and range) of individual color channels. The rabbit 388 anti-ChAT and Western images were made from scanned slides, imported into Adobe Photoshop as TIFF files.

RESULTS

Isolation and characterization of antibodies

We previously generated and used antibodies against *C. elegans* ChAT and VAChT to show coexpression in the nervous system, including in two specific neurons in the pharynx (Alfonso et al., 1993). In the present study, we have generated more robust monoclonal or polyclonal antibodies and have used these antibodies to identify cholinergic neurons in the body of *C. elegans* (Fig. 1). Immunization of numerous animals with a ChAT-MBP fusion protein led to the generation of five monoclonal antibodies (MAbs 1401, 1402, 1414, 1415, 1432) that recognize ChAT on Western blots and in fixed tissue. Immunizations with VAChT peptides or VAChT-MBP fusion protein led to the generation of four polyclonal sera (R383, C94, C95, C96) and one monoclonal antibody (MAb1403) that recognize VAChT on Western blots and in fixed tissue.

Several lines of evidence indicate that these antibodies are specific. 1) Staining in the nervous system was eliminated when the antibodies were preincubated with the appropriate fusion protein. 2) Immunoreactivity with anti-VAChT antibodies was decreased in specific mutant alleles of *unc-17*, whereas anti-ChAT staining was decreased in specific *cha-1* mutants. 3) Both anti-VAChT and anti-ChAT immunoreactivity were increased in transgenic strains carrying cosmid F57G7, which contains the *unc-17* and *cha-1* genes (Fig. 1). 4) Anti-VAChT antibodies recognized VAChT-MBP fusion protein but not ChAT-MBP fusion protein on Western blots, whereas anti-ChAT monoclonal antibodies recognized ChAT-MBP but not VAChT-MBP on Western blots. (Polyclonal anti-ChAT antibody R388 was suitable for immunocytochemistry but did not recognize ChAT protein on Western blots.) The five monoclonal antibodies generated against ChAT apparently recognize different epitopes of the protein; when partially degraded ChAT fusion protein is separated and transferred for Western analysis; the five monoclonal antibodies recognize different size degradation products.

Both VAChT and ChAT were difficult to detect in Western blots made from whole nematode protein preparations (Fig. 2), probably because of their low abundance. The five anti-ChAT monoclonal antibodies detected a protein of the same size (although MAb1432 worked poorly); the size was consistent with that expected from the *cha-1* coding sequence, 71.5 kDa (Alfonso et al., 1994a). All anti-VAChT antibodies detected a band that was smaller than the 58-kD size expected from the *unc-17* cDNA sequence (Alfonso et al., 1993) but was consistent with the size of the

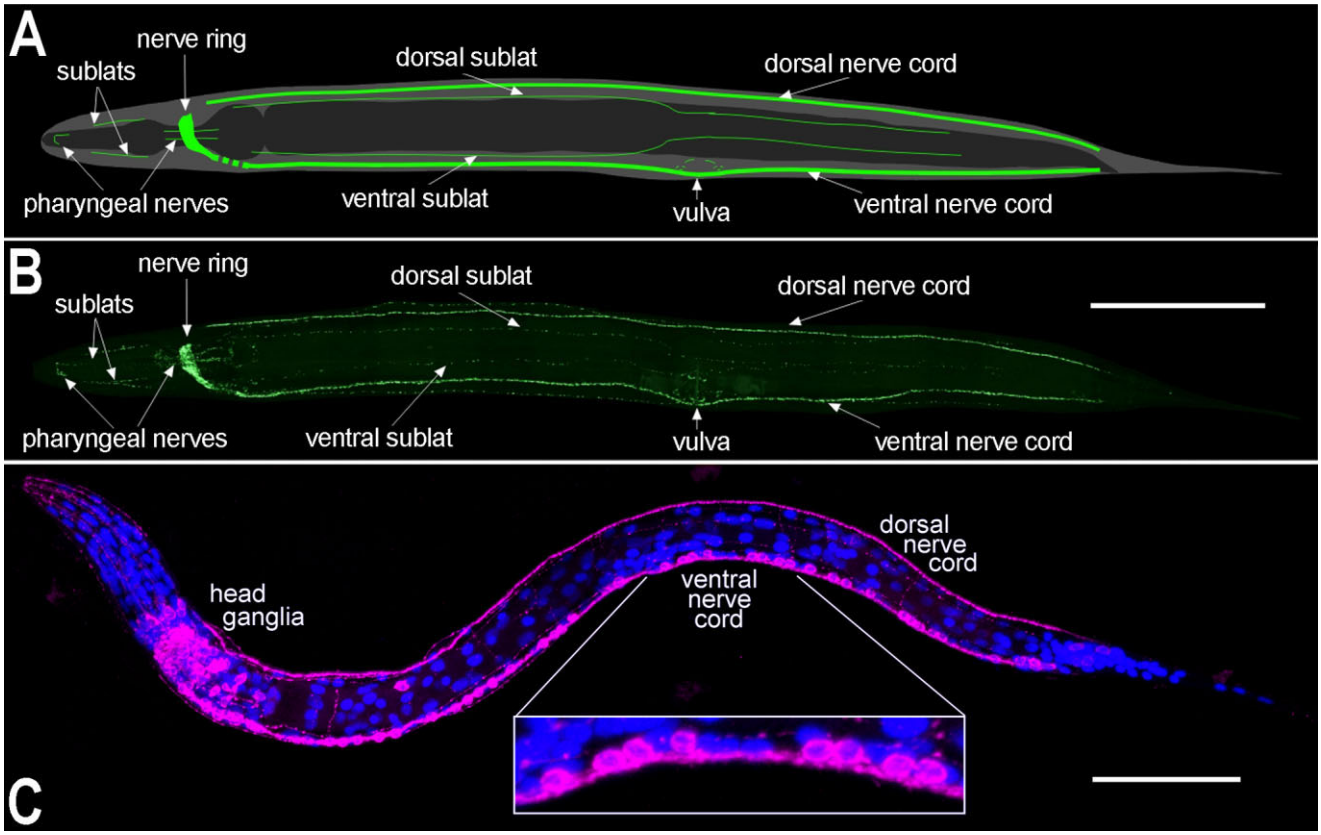

Fig. 1. The cholinergic nervous system in *C. elegans*. **A:** Diagram of an adult hermaphrodite, indicating the major nerve tracts (sublat: sublateral nerve cord). **B:** Vesicular acetylcholine transporter (VAChT) immunoreactivity (with monoclonal antibody MAb1403) in an adult hermaphrodite is punctate and is in many nerve tracts. **C:** Anti-VAChT immunoreactivity (MAb1403 in magenta) in a transgenic L3 larva overexpressing both the CHA-1 and the UNC-17 proteins; these animals have the genotype *cha-1(md39ts);mdEx8* [cosmid F57G7]. Nuclei are stained blue (with DAPI). **Inset:** Enlargement (×3) of the indicated VNC region shows a subset of cells in the VNC containing VAChT. In all images, anterior is to the left; ventral is downward. Scale bars = 100 μm in B (applies to A,B); 40 μm in C.

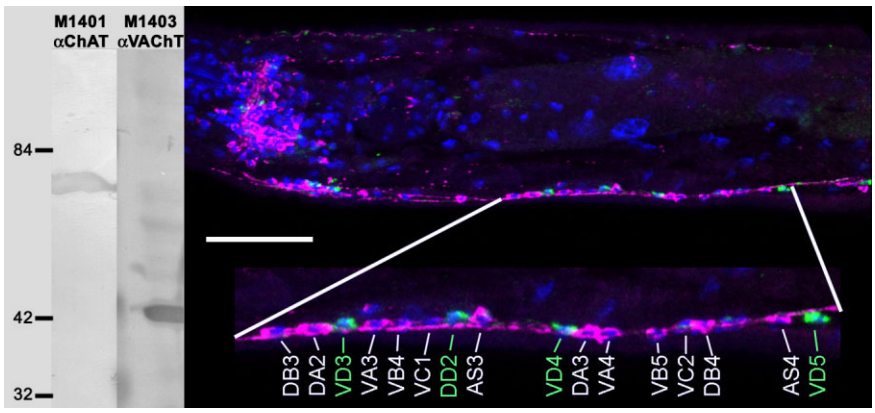

Fig. 2. ChAT and VAChT immunoreactivity on Western blots and cell identification in the ventral nerve cord in *unc-104* mutants. Left: Anti-ChAT MAb1401 (left lane) or anti-VAChT MAb1403 (right lane) was incubated with a Western membrane prepared using protein from wild-type nematodes. Anti-ChAT labels a band of ~70 kDa, whereas anti-VAChT predominantly labels a band of ~40 kDa. Right: The anterior VNC of an *unc-104;oxIs12* [*Punc-47::GFP;lin-15(+)*] adult hermaphrodite (which expresses GFP in the GABAergic neurons) was stained with anti-GFP (green), anti-VAChT (MAb1403; magenta), and DAPI (blue). Ventral cord motor neurons are either GFP positive or VAChT positive. The **inset** shows an expanded region of the nerve cord with individual neurons identified; the VC1 neuron is not noticeably VAChT positive and the VC2 neuron is only faintly VAChT positive (see text for discussion). The expanded region is 110 μm. Scale bar = 20 μm.

VAChT-MBP fusion protein expressed in *E. coli*. Higher molecular weight bands were variably detected with anti-VAChT at approximately 100 kD and 130 kD; these may represent VAChT in protein complexes (Sandoval et al., 2006).

Staining patterns of ChAT and VAChT

As reported previously, VAChT immunoreactivity is present in the nervous system in puncta that correspond to synaptic regions (Alfonso et al., 1993); the pattern was identical for all anti-VAChT antibodies. Unexpectedly, different antibodies to ChAT revealed different patterns of staining. We previously reported that a polyclonal antibody (R388) generated against a ChAT peptide smoothly labeled the processes or somas of specific neurons, including motor neurons with commissures in the body (members of the classes DA, DB, and AS) and several cells in the pharynx. These cells corresponded to cells labeled in a more punctate manner with anti-VAChT antibodies. However, we now report that, with monoclonal antibodies against ChAT fusion protein, two additional patterns of staining were observed (Fig. 3). With monoclonal antibodies 1402 and 1414, immunoreactivity was largely present in synaptic regions and was very similar to staining with anti-VAChT antibodies (Figs. 3, 4). With the three other monoclonal antibodies (MAbs 1401, 1415, 1432), staining included both punctate regions associated with synapses and smoother areas of staining in somas and processes; this staining appeared to be cytosolic or associated with the plasma membrane and other membranes. In addition, distributed punctate anti-ChAT staining was occasionally detected in nonneuronal tissues (presumably hypodermis and/or muscle) in the head; we do not know whether this immunoreactivity was specific, but ChAT activity has been detected in the hypodermis of the head of the large parasitic nematode *A. suum* (Johnson and Stretton, 1985). The three different patterns of subcellular staining of ChAT (smooth with R388; punctate with MAbs1402/1414; mixed with MAbs1401/1415/1432) all appear to be specific: they were found in the same cells in the body and tail, showed decreased staining in specific *cha-1* mutants, and showed increased staining in transgenic strains containing extra copies of the *cha-1* and *unc-17* genes (see below). Finally, all of the monoclonal antibodies recognize a protein of a similar size on Western blots of endogenous *C. elegans* proteins (Fig. 2). Our current hypothesis is that the different antibodies preferentially recognize different forms of the protein (see Discussion).

Transgenic lines containing copies of a cosmid including *cha-1* and *unc-17* sequences in either *unc-17(e245)* or *cha-1(md39ts)* mutant backgrounds were generated. These two mutants were selected as recipients for the cosmid because of their severe behavioral phenotypes and the ease of identifying transgenic rescue. The *unc-17(e245)* mutant showed decreased VAChT immunoreactivity but normal ChAT immunoreactivity (Sandoval et al., 2006). Eighteen separate lines all showed increased levels of staining with anti-ChAT (monoclonal and, when examined, R388 polyclonal) and anti-VAChT (monoclonal and polyclonal) antibodies (Fig. 1). Staining appeared to be restricted to the appropriate endogenously staining neurons but was enhanced throughout neuronal somas and processes, aiding in identification of individual cells.

The subcellular localizations of VAChT and monoclonal ChAT immunoreactivity were altered in *unc-104(e1265)* mutants (Figs. 2, 4). UNC-104 encodes a kinesin required for normal transport of synaptic vesicles and some associated proteins to synapses (Hall and Hedgecock, 1991; Otsuka et al., 1991). In *unc-104* mutants, there are large numbers of vesicles in neuronal cell bodies as well as synaptic vesicle proteins such as synaptotagmin (Nonet et al., 1993). In *unc-104* mutants, both VAChT and monoclonal ChAT immunoreactivities were more concentrated in the somas of a subset of neuronal cell bodies. However, although VAChT immunoreactivity was significantly decreased in the dorsal nerve cord and nerve ring with any of the anti-VAChT antibodies, anti-ChAT staining was still present in the nerve ring with the "smooth" (R388)

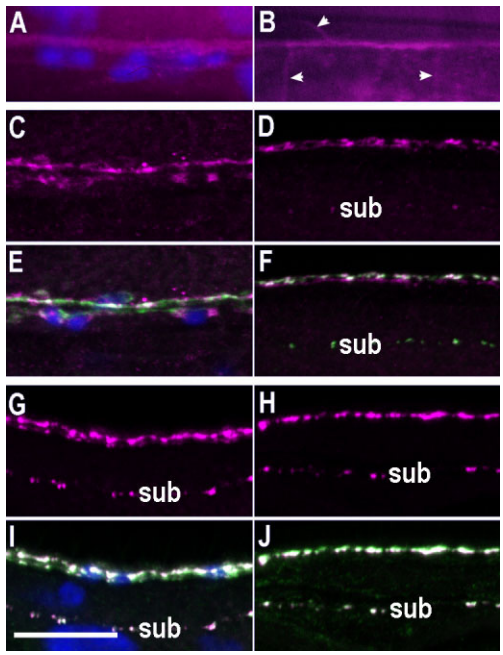

Fig. 3. The pattern of ChAT immunoreactivity in wild-type *C. elegans* varies for different antibodies. **A:** Polyclonal R388 anti-ChAT staining is faint and relatively smooth in the ventral nerve cord (magenta = ChAT and blue = nuclei). **B:** The dorsal nerve cord and some commissures (arrowheads) are evenly stained with R388 anti-ChAT. **C–F:** ChAT labeled with one of the "mixed"-pattern monoclonal antibodies, MAb1401, is more punctate than R388, but is less punctate than MAb1414 (G–J). C: MAb1401 anti-ChAT (magenta) in the ventral nerve cord. D: MAb1401 ChAT (magenta) is unevenly distributed in the dorsal nerve cord; it does not label commissures and is barely detectable in the dorsal sublateral nerve cords (sub). E: MAb1401 anti-ChAT (magenta) partially colocalizes with VAChT (green); ChAT is more abundant in the cytoplasm around some neuronal nuclei (blue). F: MAb1401 anti-ChAT (magenta) and VAChT (green) in the dorsal nerve cord are only partially colocalized. **G–J:** ChAT labeled with one of the "punctate" pattern monoclonal antibodies, MAb1414, is highly colocalized with VAChT. G: MAb1414 anti-ChAT (magenta) in the ventral nerve cord and ventral sublateral nerve cord (sub) is highly punctate. H: MAb1414 ChAT (magenta) is very punctate in the dorsal nerve cord and dorsal sublateral nerve cord; it does not label commissures. I: MAb1414 anti-ChAT (magenta) strongly colocalizes with VAChT (green). J: MAb1414 anti-ChAT (magenta) and VAChT (green) strongly colocalize in the dorsal nerve cord. The top two panels were imaged with a standard fluorescence microscope; the others were imaged with a confocal microscope. E, F, I, and J shown green C96 anti-VAChT staining and blue nuclei stained with DAPI. (Nuclei in the region of the dorsal nerve cord are not shown, because no neuronal nuclei lie within this region.) Scale bar = 10 μm.

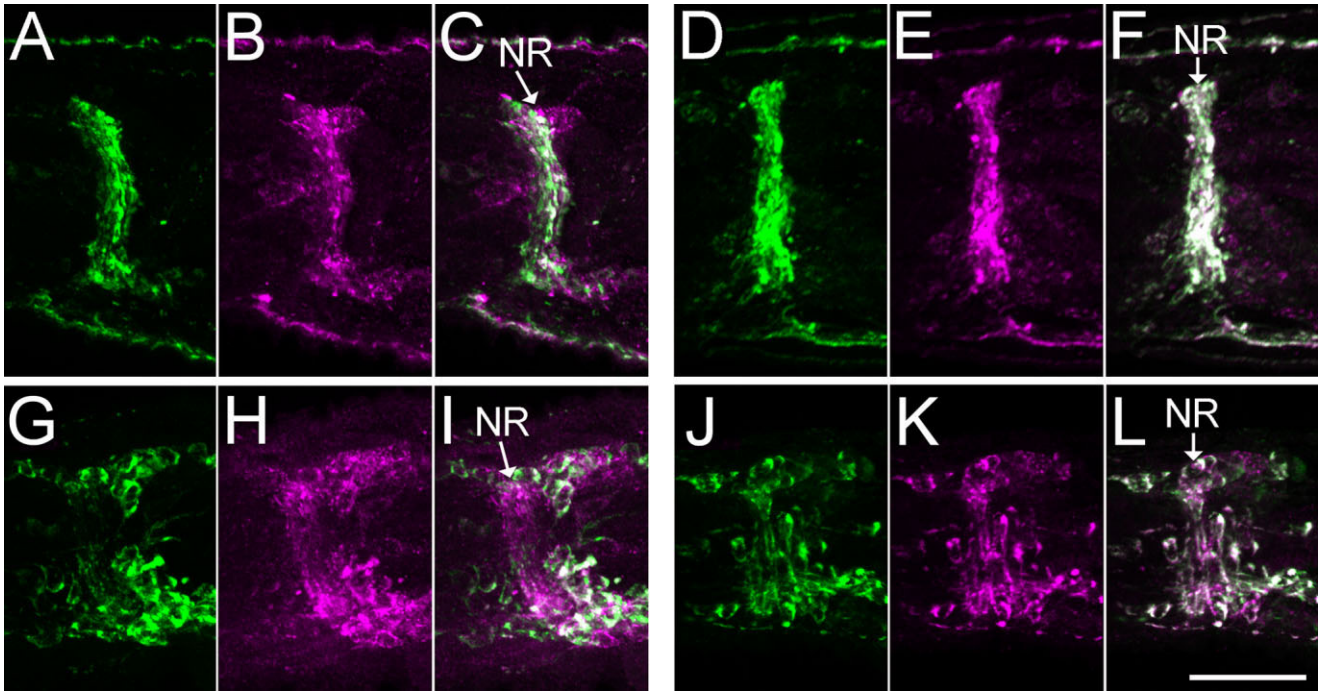

Fig. 4. ChAT and VAChT immunolocalization in wild-type animals and *unc-104* mutants. **A–C:** The nerve ring of a wild-type adult hermaphrodite was stained with anti-VAChT (A; polyclonal C96 in green) and "mixed" monoclonal antibodies to ChAT (B; MAbs 1401 + 1415 + 1432 in magenta). Both proteins are found predominantly in the nerve ring, but ChAT is less punctate and is more prevalent in neuronal somas. C: Fused image. D–F: Anti-VAChT (**D**; green) and "punctate" anti-ChAT (**E**; MAbs 1402 + 1414 in magenta) are more colocalized in the nerve ring of a wild-type hermaphrodite, although there are still some differences in relative intensity in different regions. **F:** Fused image. **G–I:** In *unc-104* mutants, many synaptic vesicles and associated proteins are mislocalized to cell somas rather than at synapses. G: Anti-VAChT (green) is found predominantly in puncta in neuronal somas, rather than in the nerve ring, whereas "mixed" anti-ChAT (H; MAbs 1401 + 1415 + 1432 in magenta) is still found in the nerve ring, as well as at higher levels in neuronal somas. I: Fused image. **J–L:** In *unc-104* mutants, anti-VAChT (J; green) and "punctate" anti-ChAT (K; MAbs 1402 + 1414 in magenta) are found predominantly in the same neuronal somas. L: Fused image. The location of the nerve ring (NR) is shown in the fused images (C,F,I,L) and was determined by the absence of cell somas (nuclei indicated by staining with DAPI; not shown). Scale bar = 20 μm.

and "mixed" (MAbs1401/1415/1432) antibodies (Figs. 3, 4). In addition, the location of the puncta within the somas was not identical for anti-VAChT and anti-ChAT antibodies (Fig. 4). This suggests that both VAChT and at least one form, but not all forms, of ChAT are associated with synaptic vesicles or are transported by UNC-104.

Identification of cholinergic neurons in the ventral nerve cord

In total, 75 VNC motor neurons have somas in the VNC or adjacent ganglia: 58 in the VNC proper, 10 in the retrovesicular ganglion at the anterior portion of the VNC, and 7 in the preanal ganglion at the posterior portion of the VNC. These motor neurons predominantly innervate the dorsal or ventral body wall muscle and have been assigned to eight classes by birth time and location, direction of extension of processes, and patterns of connectivity (White et al., 1976, 1986). For the present study, we have limited our analysis to the 65 motor neurons with somas in the VNC proper and the preanal ganglion. On the basis of the cell body staining observed in the transgenic (overexpressing) strains (Fig. 1), *unc-104* mutants (Figs. 2, 4), and wild-type animals, we have identified 49 of these 65 "ventral cord" motor neurons (45 in the VNC proper and 4 in the preanal ganglion) as putative cholinergic neurons (see Table 1).

TABLE 1. Cholinergic Neurons[1]

Name	No. of cells	Cell type	Notes
ALN	2 in LG	Interneurons	Weak and variable
AS	9 in VNC (AS2-10) 1 in PAG (AS11)	Motor neurons	
DA	6 in VNC (DA2-7) 2 in PAG (DA8-9)	Motor neurons	
DB	5 in VNC (DB3-7)	Motor neurons	
DVC	1 in DRG	Interneuron	
HSN	2 at vulva	Motor neurons	Stains in adult
PLN	2 in LG	Interneurons	Weak and variable
PVC	2 in LG	Interneurons	Weak and variable
PVP	2 in PAG	Interneurons	
PVW	2 in LG	Interneurons	Weak and variable
SDQ	2 in body	Interneurons	
VA	10 in VNC (VA2-11) 1 in PAG (VA12)	Motor neurons	
VB	9 in VNC (VB3-11)	Motor neurons	
VC	6 in VNC	Motor neurons	Stains in adult; VC1,2,6 often weak
Total motor neurons	45 in VNC 2 at vulva 4 in PAG = **51 total**		
Total interneurons	2 in body 2 in PAG 8 in LG 1 in DRG = **13 total**		

[1]In addition to the cells listed, we currently estimate that there are approximately 55 cholinergic neurons (in hermaphrodite adults) not listed in here. These include 7 "ventral cord motor neurons" with cell bodies in the retrovesicular ganglion, ~20 cells with cell bodies in the head and projections in the sublateral nerve cords (including the anterior sublateral nerve cords), 7-10 cells in the pharynx, and ~20 additional cells in the head ganglia. PAG, preanal ganglion; DRG, dorsorectal ganglion; LG, lumbar ganglia.

The *unc-104* mutant and transgenic strains were needed for proper identification of the neurons, because many neurons have overlapping synapses, so localization of synapses is not sufficient for cell identification. On the other hand, the locations of the somas of motor neurons in the VNC are predictable (but not quite unique); analysis of somatic staining in transgenic strains and strains containing *unc-104* allowed specific cell identification. For confirmation, we used reporter strains that express GFP in specific neurons (*mgIs21*, Hobert et al., 1998; *oxIs12*, McIntire et al., 1997). The reporters were crossed into an *unc-104* background or into a transgenic strain that overexpressed *cha-1* and *unc-17*, and then immunocytochemistry was used to localize GFP and VAChT or ChAT (Fig. 2).

The cholinergic neurons in the VNC of the adult hermaphrodite are members of six different motor neuron classes: AS, DA, DB, VA, VB, and VC (White et al., 1976, 1986). DA and DB are two of the three embryonically derived classes of motor neurons; they innervate dorsal body wall muscles in first stage (L1) larvae (Sulston, 1976). Both classes are immunoreactive for VAChT and ChAT in L1 animals and in all subsequent stages (Fig. 1). The DA and DB neurons may also express the choline reuptake transporter *cho-1* (Okuda et al., 2000; Matthies et al., 2006). The third embryonic motor neuron class is the DD neurons; they initially innervate ventral muscle and subsequently rewire to innervate dorsal muscle (White et al., 1976). These neurons were previously identified as containing GABA at all stages (McIntire et al., 1993); there is no overlap between these GABAergic cells (identified by GFP expression in *unc-104;oxIs12* [*Punc-47::GFP; lin-15(+)*]; McIntire et al., 1997) and VAChT- and ChAT-expressing cells (Fig. 2).

At the end of the first larval stage (L1), five additional classes of motor neurons (AS, VA, VB, VC, and VD) are born and migrate into the VNC (Sulston, 1976). The location of the somas in the VNC of adults is slightly variable because of differences in the relative positions of the embryonic and larval neuronal somas. Making the plausible assumption that members of the same motor neuron class use the same neurotransmitter, as has been shown in *A. suum* (Johnson and Stretton, 1985, 1987), we have determined that the four non-GABAergic classes of postembryonically derived motor neurons, AS, VA, VB, and VC cells, are positive for ChAT and VAChT and are thus presumably cholinergic (Fig. 2). Thus, all of the motor neurons with cell bodies in the VNC appear to be either cholinergic or GABAergic.

ChAT and VAChT expression in ventral cord cholinergic neurons

At hatch, all 16 of the cholinergic motor neurons in the VNC and adjacent ganglia (9 DA neurons and 7 DB neurons) show similar levels of ChAT and VAChT immunoreactivity. Neurons that arise during the last part of the first larval stage are born slightly laterally and migrate into the VNC; some of these neurons become immunoreactive shortly thereafter. The absolute level of protein expression cannot be directly evaluated in individual cells in wild-type animals, but, with the more somatic anti-ChAT monoclonal antibodies, immunoreactivity decreased in the posterior half of the VNC (but not in the preanal ganglion). In addition, anti-VAChT and anti-ChAT staining in the dorsal nerve cord decreased in the posterior portion of the body. In *unc-104* mutants and in overexpressing transgenic nematodes, somatic staining is similarly uneven, with lower protein levels in a subset of somas in the posterior VNC. Occasionally, individual AS, VA, or VB somas do not show appreciable immunoreactivity.

The VC neurons, unlike the AS, VA, and VB neurons, often do not show ChAT or VAChT immunoreactivity until the adult stage. This is similar to the delayed appearance of FMRFamide-related immunoreactivity in the VC neurons observed by Schinkmann and Li (1992). In addition, the adult VC4 and VC5 neurons always show bright VAChT and ChAT immunoreactivity whereas immunoreactivity in VC1-3 and VC6 is generally lower and, in some adults, may be undetectable in VC1, VC2, or VC6 (see Fig. 2). These differences in intensity may reflect fewer synapses or less protein per synapse or both. As we have reported previously, the VC4 and VC5 motor neurons are particularly immunoreactive for VAChT in adults (Duerr et al., 2001). They do not appear to show higher than average levels of anti-ChAT immunoreactivity. These neurons form arborizations at the vulva and innervate the vulval muscles (Li and Chalfie, 1990). VC4 and VC5 are also strongly positive with antibodies to the vesicular monoamine transporter and are weakly positive with antibodies to the neurotransmitter serotonin (Duerr et al., 2001).

Other cholinergic neurons in the body and the tail

In addition to the ventral cord motor neurons, there are 16 other sensory, motor, or interneurons with somas in the midbody. Cells that are immunoreactive for ChAT and VAChT include the bilateral pair of HSN neurons, which provide major innervation of the vulval muscles and the VC motor neurons (White et al., 1986). As we previously reported, the HSNs are weakly positive for ChAT and VAChT (Duerr et al., 2001). The HSNs are strongly positive with antibodies to serotonin (Desai et al., 1988) and antibodies to the vesicular amine transporter (Duerr et al., 1999). Finally, as with the VCs, they are positive with antibodies to FMRFamide-related peptides (Schinkmann and Li, 1992). Thus, the HSNs and VCs are similarly complex in their neurotransmitter phenotype (potentially aminergic, cholinergic, and peptide containing) and in their major region of innervation, the vulva. There is strong pharmacological, genetic, and behavioral evidence that HSNs and VCs and ACh and serotonin are all important for regulating egg laying in vivo (for review see Schafer, 2006).

Elsewhere in the body, SDQR (with a lateral soma in the right anterior body) and SDQL (with a lateral soma in the left posterior body) are positive for ChAT and VAChT. These are interneurons with anterior processes that run in the dorsal sublateral nerve cords to the nerve ring and synapse onto "command" interneurons involved in locomotion, such as AVA and AVB (White et al., 1986). Staining in SDQR is very intense and is significantly stronger than that in SDQL. There are eight minor nerve bundles that run through the body of *C. elegans*: two ventral sublateral nerve cords, two dorsal sublateral nerve cords, and two pairs of lateral nerve bundles (White et al., 1986; see Fig. 1). These bundles generally contain the axons of two to five neurons whose cell bodies lie laterally in the body, in the head, or in the tail. We have found regular punctate staining with anti-ChAT and anti-VAChT in the four sublateral nerve cords. At least some of this punctate staining

may be due to SDQL and SDQR as well as the ALN interneurons or PLN interneurons (discussed below). A description of the identity and an electron microscopic description of the connectivity of the cells with synapses in the sublateral nerve cords are currently in progress (Duerr, Hall, and Rand, unpublished results).

The tail of *C. elegans* contains 39 neurons in 4 ganglia: the preanal ganglion, the dorsorectal ganglion, and a pair of lumbar ganglia (White et al., 1986; Hall and Russell, 1991). Because the location of the neuronal somas is variable, we double stained *unc-104* mutants with anti-UNC-86 (Finney and Ruvkun, 1990) and anti-VAChT antibodies to confirm cell identifications. We also constructed and examined *unc-104* strains expressing GFP in DVA (*Pdbl-1::GFP*; Suzuki et al., 1999); PHA, PHB, PHC, and PVP (*Pida-1::GFP*; Zahn et al., 2001); PHA and PVQ (*Plin-11::GFP*; Hobert et al., 1998); ALN, LUA, and PVC (*Pser-2::GFP*; Tsalik et al., 2003); or DVB (*Punc-47::GFP*; McIntire et al., 1997).

The preanal ganglion is formed as a swelling of the posterior part of the VNC. It contains the somas of 12 neurons, 7 members of the major motor neuron classes (AS, DA, DD, VA, and VD as described above), 3 interneurons, and 2 additional motor neurons. The motor neuron classes that appear cholinergic in the VNC also appear cholinergic in the preanal ganglion: two DAs, one AS, and one VA. In addition, the pair of PVP interneurons appears to be cholinergic. These embryonic neurons extend anterior processes in the VNC to the nerve ring, where they form a number of synapses (White et al., 1986; Hall and Russell, 1991). The processes of the left PVP and left PVQ help establish the left VNC tract (Garriga et al., 1993).

The dorsorectal ganglion contains the somas of 3 interneurons, whereas the right and left lumbar ganglia each contain 12 neural somas: 5 sensory neurons and 7 interneurons. One of the dorsorectal interneurons, DVC, appears to be cholinergic (as identified by its position and lack of GFP in *Punc-47::GFP*- and *Pdbl-1::GFP*-containing strains). In the lumbar ganglion, the bilateral ALN interneurons and bilateral PLN interneurons weakly and variably express VAChT and ChAT (identified by their position and expression of *Pser-2::GFP* or UNC-86). These interneurons send posterior processes that may have some sensory function as well as anterior processes in the dorsal (ALN) and ventral (PLN) sublateral nerve cords to the nerve ring. These anterior processes may contribute to the punctate ChAT and VAChT immunoreactivity present in these nerve cords (Duerr, Hall, and Rand, unpublished results). In addition, the bilateral pair of PVC interneurons (in the lumbar ganglia) is occasionally positive for ChAT and VAChT. These neurons have anterior processes with synaptic output in the VNC and nerve ring; they are important for controlling forward movement (White et al., 1986; Hall and Russell, 1991). Finally, the bilateral pair of PVW neurons, which send anterior processes in the VNC but contain few synapses (Hall and Russell, 1981), is weakly immunoreactive. We note that none of the cells clearly identified as sensory neurons in the tail (PHA, PHB, PHC, PQR, PVR, and PLM; see WormAtlas, 2006) appear to be cholinergic.

In *unc-104* mutants and in transgenic strains overexpressing ChAT and VAChT, the immunoreactivity in somas in the tail is more variable than in the ventral nerve cord somas. However, the pattern of synaptic expression in processes in the tail in wild-type nematodes is quite consistent. Therefore, our current hypothesis is that the variability in somatic staining in *unc-104* mutants and overexpressing strains does not reflect actual differences in the identity of the cholinergic cells but rather is due to variably low levels of cholinergic protein expression in cell somas; under some circumstances, the expression levels are low enough that they are close to the sensitivity limits of our immunohistochemical methods.

Other nematode species

We have assayed the ability of our panel of antibodies to recognize putative cholinergic neurons in related nematode species. Several of the antibodies recognize antigens with a distribution expected for ChAT or VAChT in *C. briggsae*, *C. remanei*, and *Pellioditis* sp. In the more distantly related parasitic nematode *A. suum*, one of the polyclonal antibodies to VAChT (C96) and three of the monoclonal antibodies to ChAT recognize proteins present in a subset of neurons in the nervous system (Duerr, Johnson, and Rand, unpublished observations). These labeled neurons include neurons that are known from biochemical evidence to contain ChAT activity (Johnson and Stretton, 1985).

DISCUSSION

Cholinergic neurons in *C. elegans*

These studies provide a description of the cholinergic neurons in the body and tail of *C. elegans*; expression in the head will be described in a future communication. Among the 113 neuronal somas in the body and tail, 70 are motor neurons, and 51 of these 70 are positive for ChAT and VAChT (Table 1). These include two of the three major classes of body motor neurons in the L1 larva and six of the eight major classes in the adult ventral nerve cord. Among the other 19 motor neurons, 17 are GABAergic (McIntire et al., 1997). Among the 27 interneurons with somas in the body and tail, 12 interneurons are positive for ChAT and VAChT (many only weakly). In contrast, none of the 16 neurons that have been clearly identified as sensory neurons in this region contains ChAT or VAChT immunoreactivity (although the two ALN and two PLN neurons may be both interneurons and sensory neurons). Thus, ACh appears to be predominantly a neuromuscular transmitter in *C. elegans*. However, it is important to note that motor neurons in *C. elegans* often form synapses onto other neurons (especially other motor neurons) and therefore have some of the properties of interneurons (White et al., 1986).

It has been clear for many years that muscle in the large nematode *A. suum* responds to ACh and cholinergic agonists (del Castillo et al., 1963). The *A. suum* motor neurons corresponding to the *C. elegans* classes DA, DB, and AS (DE3, DE2, and DE1, respectively) were shown by electrophysiological analysis to be excitatory (Walrond et al., 1985), and direct enzymatic assay indicated that these same neurons contained ChAT activity (Johnson and Stretton, 1985). The immunolocalization studies presented above for *C. elegans* are completely consistent with the *A. suum* data; they provide additional evidence that DA, DB, and AS motor neurons are cholinergic. These results are also consistent with the finding that ionotropic ACh receptors or subunits are expressed in *C. elegans* body muscles (for review see Francis et al., 2003; Brown et al., 2006).

In addition to the intense immunoreactivity in the ventral and dorsal nerve cords (predominantly from motor neurons with somas in the VNC), there is regular punctate staining in the dorsal and ventral sublateral nerve cords. Some of the immunoreactivity in the sublateral nerve cords is likely to be due to the processes of the pairs of SDQ interneurons (whose somas lie laterally in the body) and the ALN and PLN interneurons (whose somas are in the tail). Preliminary evidence suggests that some interneurons of the head also have cholinergic processes in the sublateral nerve cords (Duerr, Hall, and Rand, unpublished observations).

Other immunoreactive cells include the pair of HSN motor neurons and tail interneurons DVC, PVPL, and PVPR, and the two PVC and two PVW interneurons are weakly and variably immunoreactive. Although the weak and variable cholinergic expression in the PVW neurons might be related to their paucity of synapses, no such correlation is apparent with the PVC cells: they have significant synaptic output both in the ventral nerve cord and in the nerve ring (White et al., 1986). It is conceivable that the PVC neurons use an additional, more strongly expressed neurotransmitter; the PVC cells have not been observed to contain GABA, dopamine, or serotonin.

Subcellular localization of ChAT

Despite decades of study, there is still uncertainty about the precise subcellular location of ChAT activity in neurons. In *D. melanogaster*, ChAT immunoreactivity was present predominantly in neuropil, and ChAT appeared to be membrane associated (Pahud et al., 1998). Immunoelectron microscopic studies of ChAT distribution in vertebrates have yielded contradictory results, including 1) cytosolic distribution, 2) association with most membranes, or 3) localization in cytosol and also in association with synaptic vesicles and plasma membrane (for review see Oda, 1999). Biochemical studies indicated that most ChAT activity in mammals was soluble. However, studies also indicated that newly synthesized ACh was preferentially loaded into synaptic vesicles and released, implying spatial proximity between the sites of ACh synthesis and vesicular transport (Collier and Katz, 1974). Under some conditions, ChAT activity was purified in association with membranes, including synaptic vesicles, and ChAT localization may be altered by phosphorylation or GPI anchors; unfortunately, the significance of these findings is still controversial (Oda, 1999).

In *C. elegans*, biochemical studies found more than 90% of ChAT activity to be soluble, although a small amount required detergent extraction (Rand and Russell, 1985). We have now found different subcellular immunolocalization patterns of ChAT in *C. elegans*; one pattern is fairly uniform (R388), one is punctate (MAbs 1402, 1414), and one is mixed (MAbs 1401, 1415, 1432). All of these antibodies appeared to be specific for ChAT: staining was eliminated by preincubation with the immunogen, was decreased in particular *cha-1* mutant strains, and was increased in transgenic lines that overexpress ChAT. In addition, all of the monoclonal antibodies recognize a similar size protein on Western blots of nematode proteins. This specificity suggests that the different patterns might all be "correct," and might reflect alternative states of the ChAT protein. We therefore favor a model in which there are at least two conformations of the protein present (in all species), one soluble and relatively uniformly distributed and the other associated with membranes, especially at synapses. The conformation associated with membranes would be mislocalized in *unc-104* mutants (which have a defect in vesicle transport). In *C. elegans*, the forms might differ by covalent modification and/or association with other proteins, but there would be at least some epitopes present on the vesicle-associated ChAT that are missing or inaccessible on cytoplasmic ChAT. Thus, antibody preparations that recognize such epitopes would reveal a synaptic location for the enzyme, whereas antibodies recognizing other parts of the ChAT protein would reflect the total distribution of ChAT, and the small amount of synaptic ChAT would be masked by the abundance of soluble ChAT.

Complexity in the localization of neurotransmitter synthetic enzymes is found for at least two other enzymes; both tyrosine hydroxylase (dopamine synthesis) and L-glutamic acid decarboxylase (GABA synthesis) occur in membrane-associated and soluble forms (McGeer et al., 1971; Kuczenski and Mandell, 1972; Reetz et al., 1991; Kanaani et al., 1999). In addition, newly synthesized dopamine (Chen et al., 2003) and GABA (Jin et al., 2003) are preferentially loaded into synaptic vesicles for release. Thus, association of an active form of neurotransmitter synthetic enzymes with synaptic vesicles and transporters may be a general feature of neurons that release classical neurotransmitters.

In contrast to the case with ChAT, immunoreactivity with all anti-VAChT antibodies appears punctate in wild-type animals and is severely mislocalized in *unc-104* mutants. This is consistent with VAChT being an integral membrane protein of synaptic vesicles and is consistent with the pattern of VAChT immunoreactivity at the electron microscopic level in mammals and *D. melanogaster* (Gilmor et al., 1996; Yasuyama et al., 2002).

Regulation of cholinergic proteins

The *cha-1* and *unc-17* genes are uniquely organized: their messages, which share no coding sequence, are produced by alternative splicing (Alfonso et al., 1994b). The genes share a promoter and the first noncoding exon, with the rest of the *unc-17* exons contained within the first intron of *cha-1*. This overall organization is conserved in *D. melanogaster* and mammals; its uniqueness and preservation through evolution suggest a conserved function (Eiden, 1998). In most of the ventral cord cholinergic neurons of *C. elegans*, the relative levels of VAChT and ChAT immunoreactivity appear similar. However, levels of expression are disparate in several neurons in the head (ChAT generally more abundant) and in the VC4 and VC5 neurons in the body (VAChT more abundant). We do not know how these apparent differences arise; the two genes could be differentially transcribed or spliced in some cells or there might be differences in posttranslational processes. Future examination of the regulatory sequences in this locus may indicate possible sources of variable regulation. In mammals, ChAT is present in nonneuronal cells that lack VAChT; in these cells, ACh appears to be actively exported from the cytosol via a member of the organic cation transporter (OCT) family (for review see Racke et al., 2006). Nonvesicular release of ACh might also be important for axon guidance (Yang and Kunes, 2004). OCT family members have been identified in *C. elegans* (for review see Eraly et al., 2004), but it is not

known whether they have the correct specificity and location to transport ACh.

In addition to variations in the relative expression of ChAT vs. VAChT in specific cells, the overall level of both proteins appears to vary consistently, with lower levels of expression in many of the posterior VNC motor neurons (but high levels in the preanal ganglion). This is similar to studies in *A. suum* (Johnson and Stretton, 1985) reporting that ChAT enzymatic activity in posterior motor neurons was significantly lower than that in anterior motor neurons. Similarly, the anterior SDQR is more immunoreactive than the posterior SDQL. Finally, immunoreactivity for both ChAT and VAChT is weak and variable in many of the cells in the tail.

The cholinergic markers described in this paper are already proving useful for analyzing the development and morphology of cholinergic neurons in *C. elegans*. The putative cholinergic synapses visualized with these markers are distributed throughout the body at all developmental stages. These antibodies can be readily used to screen for and study mutants with altered identities, morphologies, or patterns of connectivity of cholinergic neurons and to examine the role of subcellular localization of ChAT in cholinergic function.

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