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Identification and Characterization of *Drosophila* Genes for Synaptic Vesicle Proteins

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Proteins associated with synaptic vesicles are likely to control the release of neurotransmitter. Because synaptic transmission is fundamentally similar between vertebrates and invertebrates, vesicle proteins from vertebrates that are important for synaptic transmission should be present in *Drosophila* as well. This investigation describes *Drosophila* homologs of vamp, synaptotagmin, and rab3 that are expressed in a pattern consistent with a function in *Drosophila* neurotransmission. One previously reported candidate (*syb*), a *Drosophila* homolog of the vamp or synaptobrevin proteins, has been shown to be expressed at very low levels in neurons and is most abundant in the gut. A neuronal *Drosophila* vamp (*n-syb*) is described here and is localized to chromosome band 62A. Northern analysis and *in situ* hybridizations to mRNA indicate that the novel vamp, as well as the genes for synaptotagmin (*syt*) and rab3 (*drab3*), is expressed in the *Drosophila* nervous system. These genes are widely (perhaps ubiquitously) expressed in the nervous system and we have no evidence of additional neuronal isoforms of synaptotagmin, vamp, or rab3. Immunoreactivity for synaptotagmin and vamp is located in synaptic regions of the nervous system. This distribution suggests that these molecules are components of synaptic vesicles in *Drosophila*. The conserved structure and neuronal expression pattern of these genes indicate that they may function in processes that are required for both vertebrate and invertebrate synaptic transmission. Because of their distribution in the nervous system and because *n-syb, synaptotagmin,* and *drab3* do not appear to be in a family of functionally redundant homologs, we predict that mutation of these genes will have a profound neurological phenotype and that they are therefore good candidates for a genetic dissection in *Drosophila*.

[Key words: synaptic vesicles,* Drosophila, *vamp, synaptobrevin, synaptotagmin, p65, rab3]

Neurotransmitter release is mediated by the fusion of synaptic vesicles with the nerve terminal membrane. Synaptic vesicles must be synthesized and transported down the axon; they must also be loaded with transmitter and docked at the presynaptic membrane before they fuse with that membrane in response to elevated Ca^{2+} and release their contents into the synaptic cleft. Subsequently, they reform in the nerve terminal (Sudhof and Jahn, 1991; Trimble et al., 1991). While the molecular mechanisms underlying these processes are not understood, proteins associated with synaptic vesicles are probably involved at each step.

In recent years a great deal of progress has been made in identifying proteins in the synaptic vesicle membrane. The genes have been cloned and the primary structures determined for several of these proteins, including synaptophysin (Buckley et al., 1987; Leube et al., 1987; Sudhof et al., 1987), SV2 (Bajjalieh et al., 1992; Feany et al., 1992), synaptotagmin/p65 (Perin et al., 1990; Wendland et al., 1991), vamp/synaptobrevin (Trimble et al., 1988; Elferink et al., 1989), rab3 (Touchot et al., 1987; Matsui et al., 1988), and synapsin (McCaffery and DeGennaro, 1986; Schiebler et al., 1986). Although biochemical and structural data have given some clues, the role these proteins play in the life of the vesicle is unknown. Three of these proteins are investigated here.

Synaptotagmin is an integral membrane protein with a short intravesicular tail, a single transmembrane domain, and two cytoplasmic repeats with homology to the C2 regulatory domain of protein kinase C (Perin et al., 1990). Biochemical data implicate synaptotagmin in vesicle docking via its association with syntaxin and N-type Ca^{2+} channels (Bennett et al., 1992), with the latrotoxin receptor (Petrenko et al., 1991), and with RACKS, receptors for activated C-kinase (Mochly-Rosen et al., 1992). In addition, synaptotagmin may respond to changes in Ca^{2+}; the protein has been demonstrated to have both Ca^{2+}- and lipid-binding capabilities (Perin et al., 1990; Brose et al., 1992). Antibody and C2 domain peptide injections implicate synaptotagmin in vesicle release from PC12 cells (Elferink et al., 1993). However, it has been reported that Ca^{2+}-stimulated release of dense-core vesicles from PC12 cells appears normal in a mutant cell line that does not express the protein (Shoji-Kasai et al., 1992).

Rab3 is a low-molecular-weight GTP-binding protein. It is believed to exist in a vesicle-associated form as well as a free cytosolic form and has been reported to move between these forms when synaptosomes are stimulated (Fischer von Mollard et al., 1991). Evidence from other small GTP-binding proteins in systems such as the Golgi apparatus suggests that these forms

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may correspond to GTP- and GDP-bound forms of the molecule. These changes in the form of the protein cause activation of the molecule and are then responsible for vesicles budding from or fusing with donor or acceptor membranes (Bourne, 1988; Mellman and Simons, 1992). Rab3 may mediate similar membrane trafficking and targeting events for synaptic vesicles.

Vamp is a small protein of 115–120 amino acids with a proline-rich amino terminus, a highly conserved central domain, and a single transmembrane segment (Trimble et al., 1988). The function of vamp is unknown and it does not show homology to other protein families. The ability of tetanus toxin, an inhibitor of transmitter secretion, to proteolyze vamp suggests that vamp is essential for secretion (Schiavo et al., 1992a,b). A homolog was recently discovered in yeast (Gerst et al., 1992); thus, there may be a general cellular function for vamp in addition to the function served by its association with the synaptic vesicles. Vamp has also been observed in microvesicles in rat adipocytes, where it may be involved in the insulin-stimulated translocation of glucose transporters to the plasma membrane (Corley et al., 1992).

Drosophila homologs of synaptotagmin, rab3, and vamp (synaptobrevin) have been reported (Sudhof et al., 1989; Johnston et al., 1991; Perin et al., 1991). The gene of one of these, however, the *synaptobrevin* (*syb*) gene, is weakly expressed in the nervous system: *syb* appears to be expressed at a low level ubiquitously and most abundantly in the gut (Chin et al., 1993). The presence of vamp proteins in all vertebrate synaptic vesicles suggested that *Drosophila* is also likely to express a neuronal vamp. This article reports the cloning of a new *Drosophila* vamp gene. This gene is more likely to encode the synaptic vesicle–associated isoform than is the previously reported *syb* gene and so we are calling it *neuronal-synaptobrevin* (*n-syb*). Northern analysis and *in situ* hybridization indicate that expression of this gene and the genes *synaptotagmin* (*syt*) and *drab3* are localized to the nervous system. Furthermore, antibodies to synaptotagmin and vamp bind in synaptic regions of *Drosophila*, suggesting that the molecules are components of synaptic vesicles in the fly. These antibodies will also provide useful markers for the development of synapses and for analyzing mutations in these genes.

The physiology of synaptic transmission is very similar in *Drosophila* and vertebrates (Jan and Jan, 1976). As such, the molecular components of the release machinery are likely to be conserved. Previous reports have argued that the structural conservation of vamp/synaptobrevin, synaptotagmin, and rab3 homologs in *Drosophila* suggests that these proteins play a crucial role in synaptic vesicle physiology (Sudhof et al., 1989; Johnston et al., 1991; Perin et al., 1991). The finding that the *Drosophila* vamp homolog *syb* is a predominately non-neuronal gene demonstrates that this is not a sufficient criteria for assessing a protein's function (Chin et al., 1993). The localization of the novel vamp homolog *n-syb*, as well as *synaptotagmin* and *drab3*, to the nervous system, and the identification of synaptic synaptobrevin and synaptotagmin protein in *Drosophila* indicate that these proteins may function as synaptic vesicle proteins in an invertebrate. We intend to use *Drosophila* as a model system for understanding the functions of synaptic vesicle proteins. For this to succeed, the structural homologs in *Drosophila* of vertebrate synaptic vesicle proteins must also be functionally conserved. We find that *n-syb*, *synaptotagmin*, and *drab3* are strong candidates for genetic analysis. The evolutionary conservation of both structure and localization suggests that these three proteins have an important function in the life cycle of the synaptic vesicle.

Materials and Methods

Cloning of n-syb. An amplified λ-Zap cDNA library that had been made to randomly primed *Drosophila* head mRNA was screened at low stringency using the open reading frame (ORF) of rat vamp1 cDNA (Elferink et al., 1989) as a probe. Approximately 750,000 plaques were screened. Three clones that also hybridized to *Drosophila synaptobrevin* (*syb*) were isolated and shown by restriction mapping and partial sequencing to be independent isolates of a single gene (*n-syb*). Hybridization conditions for low-stringency screening and Southern blotting were 6× SSC (1× SSC: 0.15 M NaCl, 0.015 M Na-citrate), 5× Denhardt's (Sambrook et al., 1989), 0.5% SDS, and 200 μg/ml salmon sperm DNA at 55°C. The stringency of the final washes was 2× SSC, 0.1% SDS at 55°C. One of the three isolates (clone 8-1-2) was sequenced on both strands using the dideoxynucleotide method (Sanger et al., 1977) and Sequenase enzyme (U.S. Biochemical) and both dGTP and dITP nucleotide mixes. The clone contained the entire open reading frame and about 1 kilobase (kb) of 3′ untranslated sequence. This clone was used to rescreen the library at high stringency. A total of 900,000 plaques were screened using the same hybridization buffer at 65°C and a final wash stringency of 65°C, 0.1× SSC, 0.1% SDS; 900 positive colonies were identified. Of 20 clones analyzed, all were shown to be from the *n-syb* gene by restriction mapping. This was further confirmed by partial sequencing of 12 clones. A second clone from this later screen was then sequenced through the entire open reading frame on one strand using the same procedures as above in order to confirm the sequence. Sequence analysis was done with the GAP and BESTFIT programs.

At least two forms of *n-syb* cDNA were observed. Of 14 clones sequenced through the translation start site (with a primer complimentary to nucleotides 262–277), 12 were identical to Figure 1. However, two clones had a different 5′ end; they join the other clones at the site of an intron at nucleotide 4 of the sequence in Figure 1, that is, immediately after the ATG that forms the start codon. The alternatively spliced end contained stop codons in all frames, but no other in-frame start codons. It is possible that this transcript initiates translation at the second methionine, amino acid 56, but because the resulting protein would lack the proline head and some of the highly conserved central domain, it seems more likely that it represents a splicing intermediate that is not translated *in vivo*. The presence of such incompletely spliced transcripts is common in *Drosophila* libraries (Schwarz et al., 1990). Sequencing of the genomic clone revealed an insertion of six nucleotides at position 510. This insertion does not alter the reading frame and would encode an A and G in the amino acid sequence after G170. This addition may represent strain differences since the genomic library is Canton-S DNA while the cDNA library is Oregon-R DNA.

Cloning of synaptotagmin *and* drab3. An amplified *Drosophila* head cDNA library was screened at low stringency using the open reading frame of *Discopyge ommata* p65-A (Wendland et al., 1991) as a probe. Approximately 300,000 plaques were screened and 13 were isolated and shown to encode *synaptotagmin* by sequencing and restriction mapping. Hybridization and wash conditions were as described above for low-stringency screening. While all clones were derived from the same gene, two clones were found with alternate 5′ ends. One clone diverged between amino acids 59 and 60, while the other diverged between the second and third bases of the codon for amino acid 61. Repeated attempts to find independent isolates with these alternate 5′ ends were unsuccessful. These clones may represent other, less abundant splice variants encoding alternate amino termini, but their rarity may indicate that they are splicing intermediates. To search for members of a synaptotagmin gene family, the *Drosophila synaptotagmin* clone was used to probe the library at very low stringency. Hybridization was as above except the temperature was 37°C. The library filters were washed, allowed to expose, and rewashed through a series of four graded increases in stringency. The first wash conditions were 6× SSC, 0.1% SDS at 37°C; the second, 2× SSC, 0.1% SDS at 37°C; the third, 2× SSC, 0.1% SDS at 48°C; and the last, 2× SSC, 0.1% SDS at 65°C. Forty-eight positive colonies were identified and shown by sequencing or polymerase chain reaction (PCR) to be isolates from the same *synaptotagmin* gene. A low-stringency screen was also performed with *D. ommata* p65-C and no hybridizing clones were identified. Probes to *drab3* were obtained by PCR amplification of the gene from an adult head library using oligonucleotide primers based on the published sequence (John-

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CACATCCCGACCTGCGCCAGTGGAAGACAGTGAAAGAGGCCAAGTGCATCGTGCATAGAG   -92
TACCTTCATCCTAATCATCGACAGCCGAATTCGCTGAGGCCACCAGAGCACCACCAGCCA   -32
AACCACAGAAGCAACCACACAGCCACCAGAGCCATGGCGGACGCTGCACCAGCTGGCGAT    27
                                  M  A  D  A  A  P  A  G  D      9

GCACCACCCAATGCCGGAGCCCCGGCCGGAGAGGGCGGCGATGGCGAGATTGTGGGCGGA   87
 A  P  P  N  A  G  A  P  A  G  E  G  G  D  G  E  I  V  G  G    29

CCACACAATCCGCAGCAGATCGCGGCACAGAAGCGTCTGCAGCAGACGCAGGCGCAGGTC  147
 P  H  N  P  Q  Q  I  A  A  Q  K  R  L  Q  Q  T  Q  A  Q  V    49

GATGAGGTCGTGGACATCATGCGCACGAACGTGGAAAAGGTGCTGGAGCGCGACACGAAG  207
 D  E  V  V  D  I  M  R  T  N  V  E  K  V  L  E  R  D  T  K    69

CTGTCGGAGCTGGACGACCGTGCCGATGCCTTGCAGCAGGGTGCCTCGCAGTTTGAGCAG  267
 L  S  E  L  D  D  R  A  D  A  L  Q  Q  G  A  S  Q  F  E  Q    89

CAGGCGGGCAAGCTCAAGAGGAAATTCTGGCTCCAGAACTTGAAGATGATGATCATCATG  327
 Q  A  G  K  L  K  R  K  F  W  L  Q  N  L  K  M  M  I  I  M   109

GGCGTGATTGGCCTGGTTGTCGTGGGCATTATTGCAAATAAACTTGGACTCATAGGCGGG  387
 G  V  I  G  L  V  V  V  G  I  I  A  N  K  L  G  L  I  G  G   129

GAGCAGCCGCCACAGTACCAGTATCCCCCACAGTACATGCAGCCACCGCCGCCGCCACCG  447
 E  Q  P  P  Q  Y  Q  Y  P  P  Q  Y  M  Q  P  P  P  P  P  P   149

CAGCAGCCAGCCGGAGGACAGTCATCGCTGGTGGATGGGGCAGGAGCAGGAGACGGAGCA  507
 Q  Q  P  A  G  G  Q  S  S  L  V  D  G  A  G  A  G  D  G  A   169

GGAGGATCGGCAGGAGCTGGCGATCACGGCGGCGTGTAAGCAATCCCGCTGCCAGGACGA  567
 G  G  S  A  G  A  G  D  H  G  G  V  *                       181

AAGTTTCTCGATTAGTGTGCTCCTTCAGCGGGCGAAAACAAAGCAGAGAAGACAAAAGAA  627
AACCAAGAAAGGAAAACTCAGTTAAACTAGAAAATCTCGAGAATTGCAATGAAAACTCAA  687
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Figure 1. The nucleotide and translated amino acid sequences of the *n-syb* gene. The *boldfaced nucleotides −119 through −121* indicate an upstream stop codon preceding the start methionine. The transmembrane segment of the protein is indicated by *underlining* in the amino acid sequence. The total length of the protein is 181 amino acids.

ston et al., 1991). The PCR product was sequenced and shown to be identical to the published sequence in the open reading frame.

In situ *hybridization to* Drosophila *polytene chromosomes.* The salivary glands of third instar larvae were dissected out in *Drosophila* Ringer's solution, squashed onto slides, and fixed (Ashburner, 1989). The chromosomes were hybridized with biotin-labeled DNA probes (see below) in a 50% formamide buffer. After washing at 50°C in 2× SSC, the slides were treated with an HRP-avidin complex (DeTek kit, Enzo Diagnostics) and the signal developed using diaminobenzidine as a substrate for HRP. The chromosomes are counterstained with Geimsa at 1:20 in 0.1 M Na-phosphate, pH 6.9, and mounted in Permount (Fisher).

Isolation of genomic clones for drab3, synaptotagmin, *and* n-syb. A cosmid genomic library (Tamkun et al., 1992) was screened at high stringency (final wash conditions of 0.1× SSC, 0.1% SDS at 65°C) with probes encoding the open reading frame of either *Drosophila synaptotagmin* or *drab3*. Fourteen clones were isolated for *synaptotagmin* and were shown to be overlapping by restriction mapping and Southern analysis. One clone was isolated for *drab3*. It was shown to encode *drab3* by sequencing. A genomic clone for *n-syb* was found during a low-stringency screen of a λ-Charon 4A library (Maniatis et al., 1978) with the *syb* gene. Five to six genome equivalents were represented by the approximately 200,000 plaques screened. Final wash conditions were 1× SSC at 50°C. Fourteen hybridizing clones were isolated and analyzed. Seven were strongly positive and represented reisolates of *syb*. All of the seven remaining clones were shown to be *n-syb* by restriction mapping, by high-stringency Southern blotting with *n-syb* cDNAs, and by sequencing.

Northern analysis of gene expression. PolyA-selected mRNA was isolated from staged *Drosophila* embryos, first, second, and third instar larvae, pupae, adult heads, and adult bodies by phenol/urea extractions (Schwarz et al., 1988). Approximately 5 μg of mRNA per lane was separated in a 6.7% formaldehyde, 0.8% agarose gel (Sambrook et al., 1989). The mRNA was then transferred to Gene Screen 2 (DuPont), UV cross-linked, and hybridized at 50°C in 50% formamide, 6× SSC, 5× Denhardt's, 250 μg/ml salmon sperm DNA, and 5% SDS. The stringency of the final wash was 65°C, in 0.1× SSC, 0.1% SDS. The amount of mRNA loaded in each lane was quantified by probing each blot with rp49 (Aguade, 1988). Total RNA was isolated from timed embryo collections and each larval instar stage. The total RNA was then loaded at 20 μg per lane and blotted as above for the *n-syb* developmental Northern. Blots were washed and hybridized at high stringency. The total RNA blot shown in this article was exposed for 4 d with one intensifying screen.

In situ *hybridization to* Drosophila *whole-mount embryos and frozen sections.* Whole-mount embryos were fixed according to the protocol of Tautz and Pfeifle (1989) with slight modifications. Adult frozen sections were cut, fixed, and pretreated according to the protocol of Hafen and Levine (1986). For both whole-mounts and sections, formaldehyde was used in place of paraformaldehyde and proteinase-K was used for 6 min at 50 μg/ml in place of pronase. Probes were made from DNA templates and were labeled with digoxygenin (see below). All hybridizations were done at 42–45°C in 50% formamide, 5× SSC, 100 μg/ml salmon sperm DNA, 50 μg/ml heparin, and 0.1% Tween-20. The signal was developed using alkaline phosphatase–conjugated anti-digoxygenin antibodies and X-phosphate and NBT (Boehringer Mannheim) according to the manufacturer's protocol; reactions were stopped in PBS, 10 mM EDTA, 0.5% glutaraldehyde for *drab3* and *syt*. Specimens were then mounted in 80% glycerol.

DNA probe labeling. All library screening and Northern analysis were done using ^{32}P-labeled DNA probes. These probes were prepared by standard random priming procedures using α^{32}P-dCTP as the labeling nucleotide. Biotin-labeled probes were synthesized by nick translation using Bio-16-dUTP (Enzo Diagnostics) as the labeled nucleotide. Digoxygenin-labeled probes were synthesized by random priming using the reagents supplied in the Genius Kit (Boehringer Mannheim). The manufacturer's protocol was followed with the addition of an extra 30 μg of random hexamers (Pharmacia) per reaction. Probes were resuspended in Tris-EDTA buffer containing 0.2% SDS.

Immunocytochemistry of adult sections. Frozen sections of 10–12 μm thickness were cut and adhered to glass slides. Sections were fixed and stained according to a published protocol (Schwarz et al., 1990). The synaptotagmin primary antibody was provided by K. Miller and B. Wendland (Stanford Univ. Medical Center) and is an affinity-purified rabbit polyclonal raised to the cytoplasmic domain of rat synaptotagmin. This antibody recognizes a single band of the correct size on immunoblots of *Drosophila* lysates. It was used at a dilution of 1:5000. Preincubation of the serum with the fusion protein immunogen blocked synaptotagmin staining. The Cl 10.1 monoclonal anti-synaptobrevin

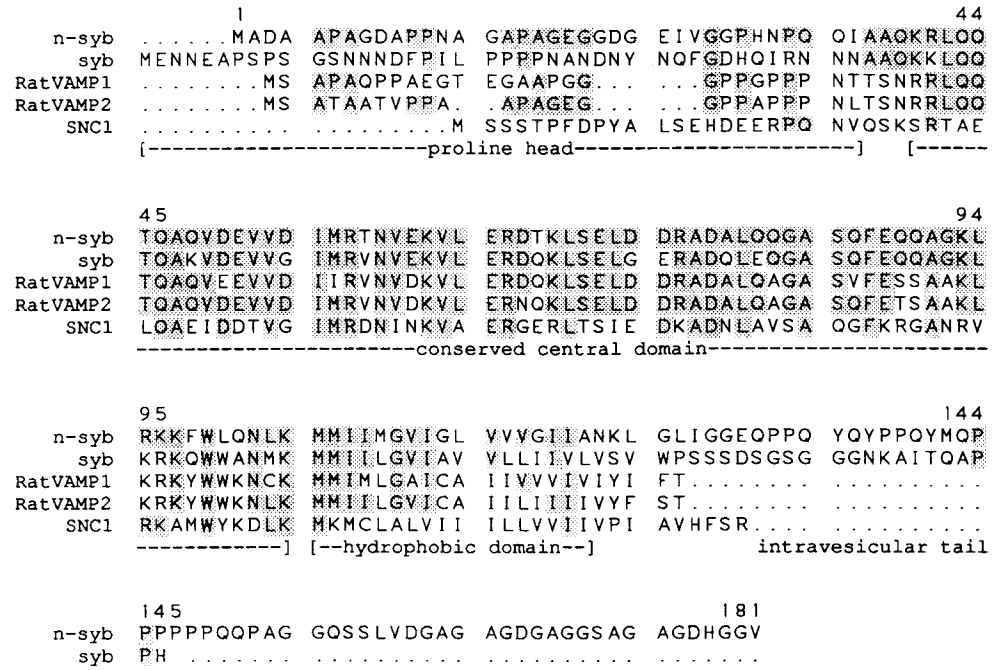

Figure 2. Homology comparison of *n-syb* to other vamp/synaptobrevin homologs. The novel *Drosophila n-syb* sequence is aligned for maximum homology with the previously reported *Drosophila syb,* rat vamp1 and vamp2, and the yeast homolog SNC1. *Shading* indicates amino acids that are identical between the *n-syb* and the previously reported sequences. The proline-rich amino terminus, the conserved central domain, the transmembrane segment, and the intravesicular region are defined below the sequences.

antibody (Baumert et al., 1989) was used at a dilution of 1:1000. This antibody recognizes a band of the correct molecular weight for *n-syb* on immunoblots of *Drosophila* head lysates. One unidentified, cross-reacting band of about 55 kDa was also detected. Signals for immunocytochemistry were developed using the ABC kit (Vector) according to the manufacturer's protocol. Sections were dehydrated in ethanol and xylene and mounted in Permount.

Results

A neuronal vamp cDNA

In a search for a neuronal homolog of vamp in *Drosophila,* three clones were purified from a *Drosophila* adult head cDNA library that was probed at low stringency with the ORF of rat vamp1. One of these was sequenced and a conceptual translation indicates it is a novel vamp homolog with an ORF of 543 bases predicting a protein of 181 amino acids (Fig. 1). A second, higher-stringency screen using the sequenced clone revealed that the gene is very abundant in the library, occurring at a frequency of nearly 1 in 1000 plaques.

The novel vamp homolog, which we have called *n-syb,* is highly homologous to previously cloned vamp proteins (Fig. 2). The structural features of the family are preserved in *n-syb*; it has a proline-rich amino terminus, a highly conserved central cytoplasmic region, and a single transmembrane segment. Through the central cytoplasmic region and transmembrane segment, *n-syb* is 70% identical to *syb* and 67% and 75% identical to rat vamps 1 and 2, respectively. The proline-rich amino terminus of *n-syb* is more similar to the rat vamps than is the equivalent region of the previously reported *syb. n-syb* has an exceptionally long intravesicular carboxyl tail and in this it resembles one of the splicing variants of *syb* (*syb-a*). While *syb* has at least one splice variant with a truncated tail (*syb-b*; Chin et al., 1993), no such isoform has been identified for *n-syb.*

We also searched for additional homologs by a low-stringency screen of a genomic library. The existence of homologous genes may frequently be more easily detected in a genomic library, since every gene should be represented equally, regardless of the level of expression of the gene or its restriction to a particular tissue or developmental stage. The *syb* gene was used as a probe to screen the genomic library at low stringency. In addition to reisolating the *syb* gene, seven more weakly hybridizing clones were also analyzed. All seven of these clones contained the *n-syb* gene; no additional members of the family were encountered. A 1.7 kb cDNA containing the ORF hybridizes to a single 8 kb EcoRI genomic fragment (Fig. 3*A*). From sequencing of genomic DNA, we determined that the ORF of *n-syb* is encoded by five exons; introns were identified at nucleotide positions 4, 153, 312, and 365 as per the numbering in Figure 1. Genomic clones can be transformed into flies and used to rescue mutants genetically. Rescue, along with locations of intron/exon boundaries, will facilitate the identification of mutants and the amplification and sequencing of genomic DNA from mutant strains to identify the site of the mutation.

In situ hybridization of *n-syb* to polytene chromosomes places the gene near the left end of chromosome 3 in the region of 62A; it is not clustered with *syb,* which is found at 46F.

cDNA and genomic clones for synaptotagmin *and* drab3

A *Drosophila* adult head cDNA library was screened at low stringency using a probe corresponding to most of the ORF of *D. ommata* synaptotagmin-A. Thirteen hybridizing clones were isolated and shown to encode a single *Drosophila* homolog of synaptotagmin. A sequence for *Drosophila synaptotagmin* (*syt*) has recently been published (Perin et al., 1991) that is nearly identical to our sequence (data not shown). Only one difference in the predicted protein sequence was found: amino acids 168 and 169, V and Q, in the published sequence are not present in the clones we isolated. The *Drosophila* adult head cDNA library was also screened at low stringency with a probe made to *Drosophila* synaptotagmin and to *D. ommata* p65-C to look for other members of a gene family; in vertebrate species (*D. ommata* and the rat), two or more isoforms of the protein have been observed (Geppert et al., 1991; Wendland et al., 1991). The synaptotagmin protein in *Drosophila,* however, appears to be the product of a single gene in *Drosophila*; we did not find any additional homologs in these screens.

To isolate genomic clones, a cosmid library (Tamkun et al.,

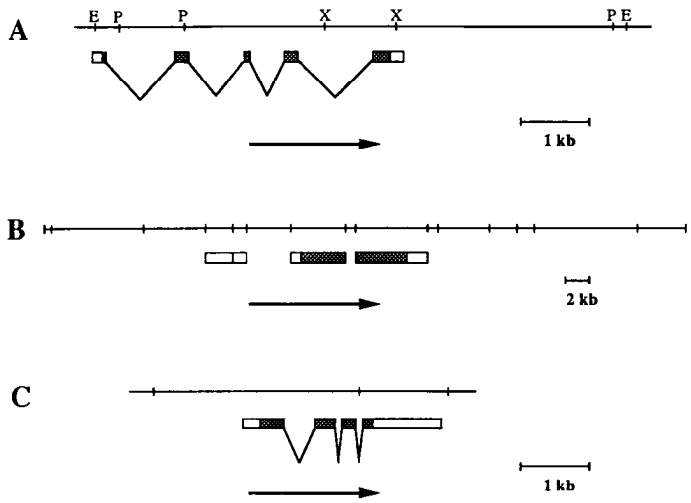

Figure 3. Partial genomic restriction maps for *n-syb* (*A*), *syt* (*B*), and *drab3* (*C*). *A*, The entire *n-syb* ORF (*shaded boxes*) is located within 5.0 kb of genomic DNA, as is at least some 5′ and 3′ untranslated sequence (*open boxes*). Introns are present at nucleotide positions 4, 153, 312, and 365 as per the numbering of the cDNA in Figure 1. Sizes of introns have not been determined. *B*, The *syt* cDNA is spread across at least 20 kb of genomic DNA. Those EcoRI restriction fragments that hybridize to a 2.2 kb *syt* cDNA are indicated by the *boxes*. Oligonucleotide probes indicate that the two EcoRI fragments at the *left* include 5′ untranslated sequence and that the EcoRI fragment at the *right* includes both ORF and 3′ untranslated sequences. The entire ORF is present within two EcoRI fragments (*shaded boxes*). *C*, The entire *drab3* ORF (indicated by the *shaded boxes*) is located within 4.3 kb of genomic DNA, as is at least some 5′ and 3′ untranslated sequence (*open boxes*). Three introns interrupt the ORF. These introns are approximately 400, 100, and 75 base pairs in length and occur at nucleotide positions 811, 930, and 1102 as per the numbering of Johnston et al. (1991). In *A* the *tick marks* on the genomic map indicate EcoRI (*E*), PstI (*P*), and XhoI (*X*) restriction sites; in *B* and *C* they indicate EcoRI restriction sites. *Arrows* indicate the direction of transcription. A size marker is included for each panel.

1992) was screened with the ORF of *Drosophila synaptotagmin* and with a probe synthesized to the ORF of the previously cloned *Drosophila drab3*. Fourteen overlapping *synaptotagmin* clones were isolated that contained either one or both of a 4.8 and a 6.5 kb EcoRI fragment that hybridized to an ORF probe. The 14 overlapping clones span more than 55 kb of genomic DNA. Probing Southern blots with a 2.2 kb cDNA that includes the entire ORF and with oligonucleotides to various regions of the *synaptotagmin* cDNA demonstrates that the cDNA is spread across at least 20 kb of genomic DNA. Genomic EcoRI fragments that hybridize to this cDNA are diagrammed in Figure 3*B*. All exons encoding the ORF are present in the 4.8 and 6.5 kb EcoRI fragments. For *drab3*, a single hybridizing cosmid was isolated. It was shown to contain the *drab3* gene by sequencing. The entire ORF is represented in two genomic EcoRI fragments of 1.3 and 3.0 kb. Subcloning, sequencing, and PCR between these fragments demonstrated that the *drab3* ORF is punctuated by three introns of approximately 400, 100, and 75 base pairs at nucleotide positions 811, 930, and 1102 as per the numbering of Johnston et al. (1991). An intron/exon map of that portion of the *drab3* gene that encodes the ORF is shown in Figure 3*C*.

Transcription of synaptotagmin, drab3, *and* n-syb

Since these *Drosophila* genes were found on the basis of homology to vertebrate proteins, and not by purification of proteins from synaptic vesicles, we have sought evidence that they are indeed neuronal and synaptic by studying their expression pattern, first with RNA blots and then by *in situ* hybridization and immunocytochemistry (see below). In addition, because the predicted phenotype of a mutation in a gene expressed only at one developmental stage or only in a subset of neurons would differ from the phenotype predicted for a gene that functioned at all synapses, characterizing the distribution pattern is an important prerequisite for genetic studies. The expression of a gene product required at every synapse would be expected to begin at 8–15 hr of *Drosophila* development, a period of extensive neurite outgrowth and synapse formation. The first known requirement for synaptic function is in the emergence of the first instar larvae from the egg case (22 hr). In later larval periods, neuronal transcripts are often hard to see in RNA blots because the brain and nerve cord are so small in comparison to muscle and digestive organs at this stage. In the pupal stage, the development of the adult nervous system requires the transcription of neuronal genes. In the adult, an approximately 10-fold enrichment of neuronal transcripts can be observed when head RNA is compared to body RNA; although the body contains peripheral neurons and the thoracic ganglia they are again small in comparison to muscle and digestive tissues.

Northern analysis of the *n-syb* transcript demonstrates that it is present in mRNA isolated from embryos, larvae, pupae, and adults in a pattern much like that described above. *n-syb* has two transcripts of 3.0 and 3.2 kb (Fig. 4). The onset of embryonic expression appears to coincide with maturation of the nervous system at about 12 hr of age, when synapses begin to form (Broadie et al., 1992). This contrasts with the homologous *syb* gene, which had been observed in very early embryos, prior to cellularization and neurogenesis (Chin et al., 1993). Prior to adulthood, the two *n-syb* transcripts appear to be present in roughly equal quantities. The adult fly expresses predominantly the 3.0 kb transcript, which is strongly enriched in the head (Fig. 4). When the same lanes were probed with *syb* (whose transcripts are 0.85 and 1.1 kb), no significant enrichment in head versus body was evident.

Northern analysis of *syt* expression showed an onset of expression that similarly coincided with synaptogenesis and an enrichment in the head relative to the body. The message exists predominantly as 4.0 and 6.5 kb transcripts in both embryos and adults (Fig. 5). The abundant, lower-molecular-weight transcript in third instar larvae is not understood, although it is consistently observed with *syt* ORF probes. A longer exposure of this blot does indicate that second and third instar larvae also express the 4.0 kb transcript.

A *drab3* probe on the same mRNA blots hybridizes to a band of approximately 3.8 kb that is probably a doublet (Fig. 6). The developmental expression pattern of *drab3* is similar to that of *syt* and *n-syb*. The transcript first appears between 12 and 15 hr and is expressed throughout development, although a longer exposure is required to detect the larval signal. *drab3* is also strongly enriched in the head compared to the body.

n-syb, synaptotagmin, *and* drab3 *mRNA are localized to the nervous system*

In situ hybridizations were performed to study further the expression patterns of each gene. We sought to determine if the gene was indeed expressed in neurons and to see if we could detect neuronal populations that did not express the gene. Such a gap in expression would imply either that the protein was not

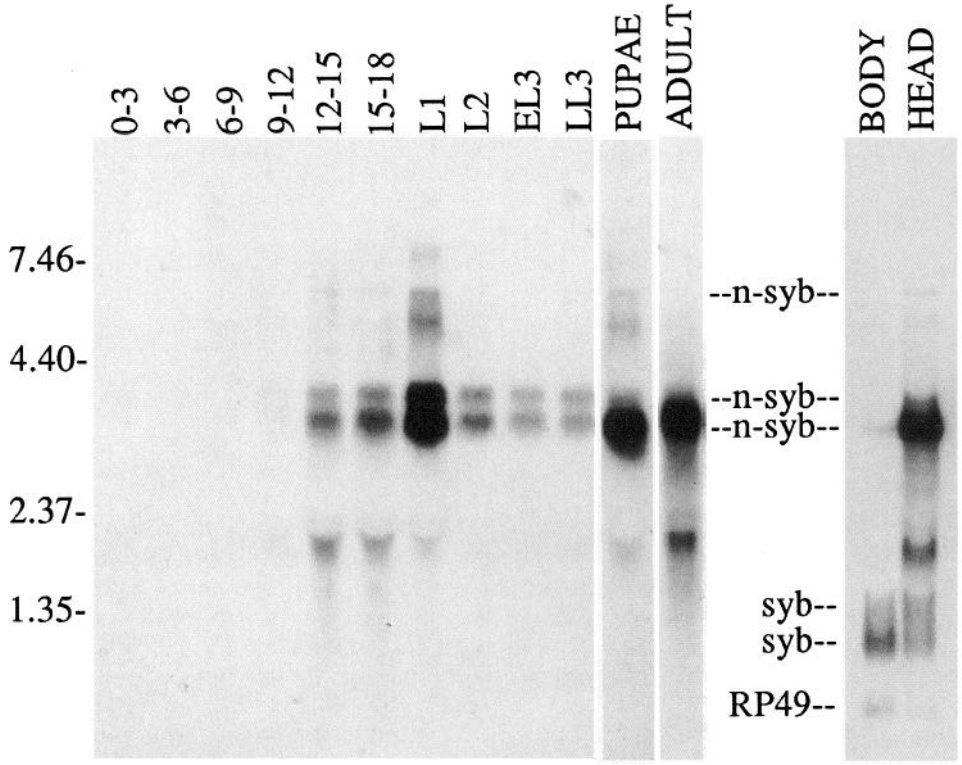

Figure 4. Expression pattern of *n-syb* determined by Northern analysis. Detectable *n-syb* expression begins at 9–12 hr of development, increases thereafter, and can be seen in all subsequent developmental stages. The transcript has two primary forms of 3.0 and 3.2 kb; the relative abundance of these forms appears to be nearly equal throughout the embryonic and larval stages. The 3.0 kb transcript predominates in the pupae and adult and is highly enriched in the head compared to the body. The head and body lanes were also probed with the *syb* gene, which shows no comparable enrichment in the head. Lanes are denoted in hours of development for embryos; *L1,* first instar larvae; *L2,* second instar; *EL3,* early third instar; *LL3,* late third instar; *ADULT,* whole adult; *BODY,* adult bodies; *HEAD,* adult heads. Lanes are loaded with 20 μg of total RNA, except the body and head lanes, which are loaded with 5 μg of polyA$^+$ mRNA. The quantitation with rp49 is indicated. The signal near 2.0 kb is an artifact caused by the exclusion of degraded *n-syb* transcript by contaminating ribosomal RNA.

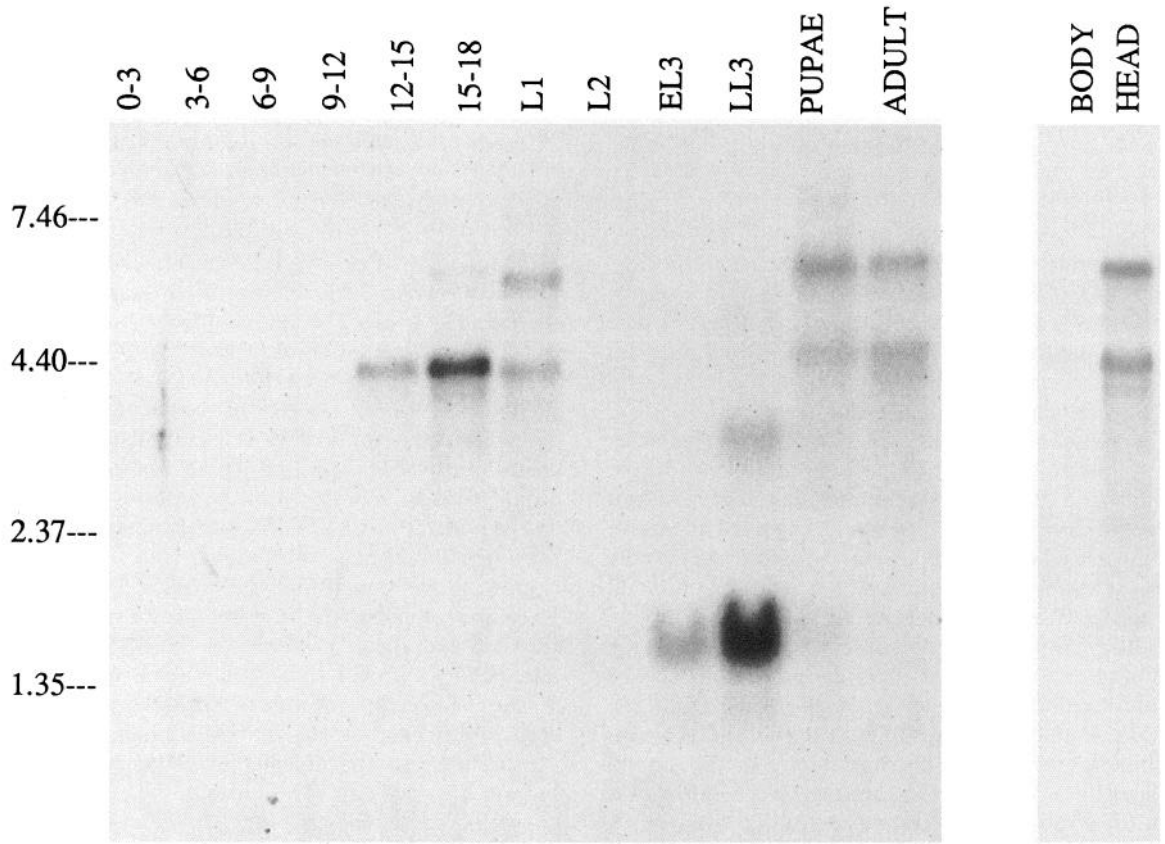

Figure 5. Expression pattern of *syt* determined by Northern analysis. Examination of the expression pattern of *syt* indicates that transcripts are present in all developmental stages and are highly enriched in the head. Transcription of *syt* begins between 9 and 12 hr of development. The two predominant transcripts in the embryo and adult are 4.0 and 6.5 kb. The faintness of the signal of the 4.0 kb transcript in second and third instar larvae (visible on longer exposures) probably reflects the extremely low percentage of larvae that is neuronal. The abundant, low-molecular-weight *syt* signal in the third instar larvae is not understood. Lanes are denoted in hours of development for embryos; other lanes are identified as in Figure 4. Lanes are loaded with 5 μg of mRNA, except the head lane, which contains approximately five times less mRNA.

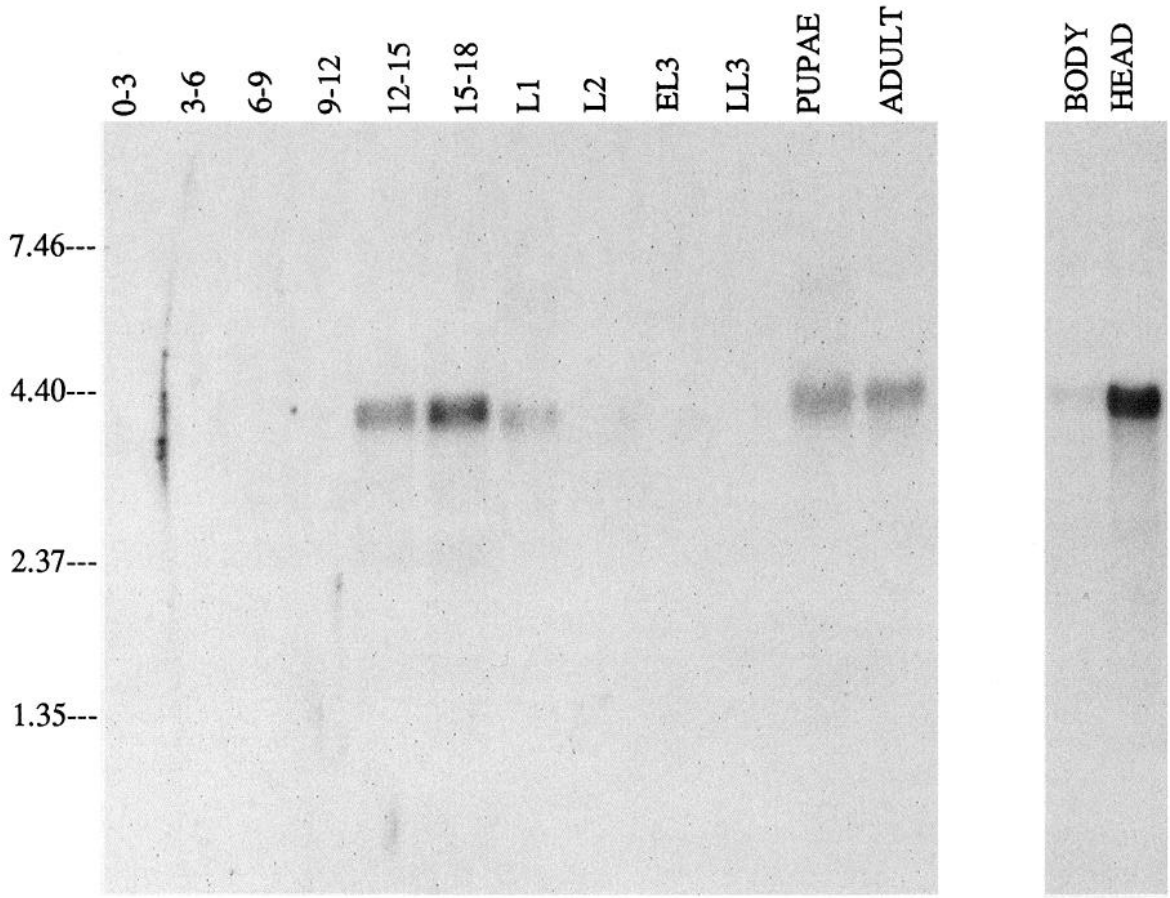

Figure 6. Expression pattern of *drab3* determined by Northern analysis. *drab3* is first expressed between 9 and 12 hr of development and is enriched in the head. The *drab3* signal appears to be a doublet at 3.8 kb. The barely detectable signal in second and third instar larvae probably reflects the extremely low percentage of larvae that is neuronal. Lanes are denoted in hours of development for embryos; other lanes are identified as in Figure 4. Lanes are loaded with 5 μg of mRNA, except the head lane, which contains approximately five times less mRNA.

required for synaptic function or that other genes are present that encode isoforms of the protein. The resolution of these *in situ* hybridizations is not capable of determining if every single neuron contains the transcript, but they might reveal if a class of neurons such as photoreceptors or cells of the embryonic nerve cord was not expressing the genes.

n-syb, synaptotagmin, and *drab3* distribution was studied in embryos and adults. The embryonic CNS consists roughly of two anterior ganglia, the sub- and supraesophageal ganglia, and the ventral nerve cord. The PNS is primarily involved in sensory function and consists of three, bilaterally symmetrical clusters of cells in most segments (Bodmer and Jan, 1987). Peripheral ganglia are also present at the anterior and posterior ends (the antennomaxillary complex and dorsal sensory cones). Sections through the head reveal the major features of the adult nervous system: photoreceptors and the optic ganglia that process the visual inputs are found at either side, and the central brain region and subesophageal ganglion lie in the middle. In the thorax, three large ganglia are present that are responsible for motor output to the flight muscles and legs and the reception of sensory information. As in other invertebrate nervous systems, the cell bodies of the central neurons form a cortical rind that surrounds the synaptic neuropil.

The expression pattern of *n-syb* was determined by *in situ* hybridization to whole-mount embryos (Fig. 7*A–D*) and adults (Fig. 7*G*). *n-syb* transcripts are first detectable at stage 13 or 14, or about 11 hr (Fig. 7*B*), in agreement with the results of the Northern analysis. The expression appears to be restricted to the nervous system, becoming quite robust in both the CNS and PNS by late embryogenesis (Fig. 7*C,D*). There were no apparent gaps of staining in the nervous system. Similarly, *in situ* hybridizations to frozen sections of adult flies indicate that the transcript is present in cell bodies of the visual system, central nuclei (Fig. 7*G*), and thoracic ganglia (not shown).

The expression of *drab3* in the embryo was examined by *in situ* hybridization of a cDNA probe to whole-mount embryos. Although the signals were weaker, the time course of *drab3* expression was similar to that of *n-syb,* beginning at about stage 13 (data not shown). In later embryos, expression is robust in the CNS and apparent in parts of the PNS, such as the antennomaxillary complex (Fig. 7*E*), but was not detected in other sensory neurons. There were no apparent gaps of staining in the embryonic CNS. In sections of the adult brain (data not shown), the staining was detectable in the cell bodies of the central nuclei but was not apparent in the cortex of the visual system. Because of the generally weak staining, it is quite possible that *drab3* is expressed in the optic ganglia and embryonic sensory neurons. Antibodies are currently being generated to examine further the distribution of drab3.

The expression of *synaptotagmin* in the embryo was also examined in whole-mount embryos and was shown to develop with a time course similar to that of *n-syb* and *drab3. Synap-*

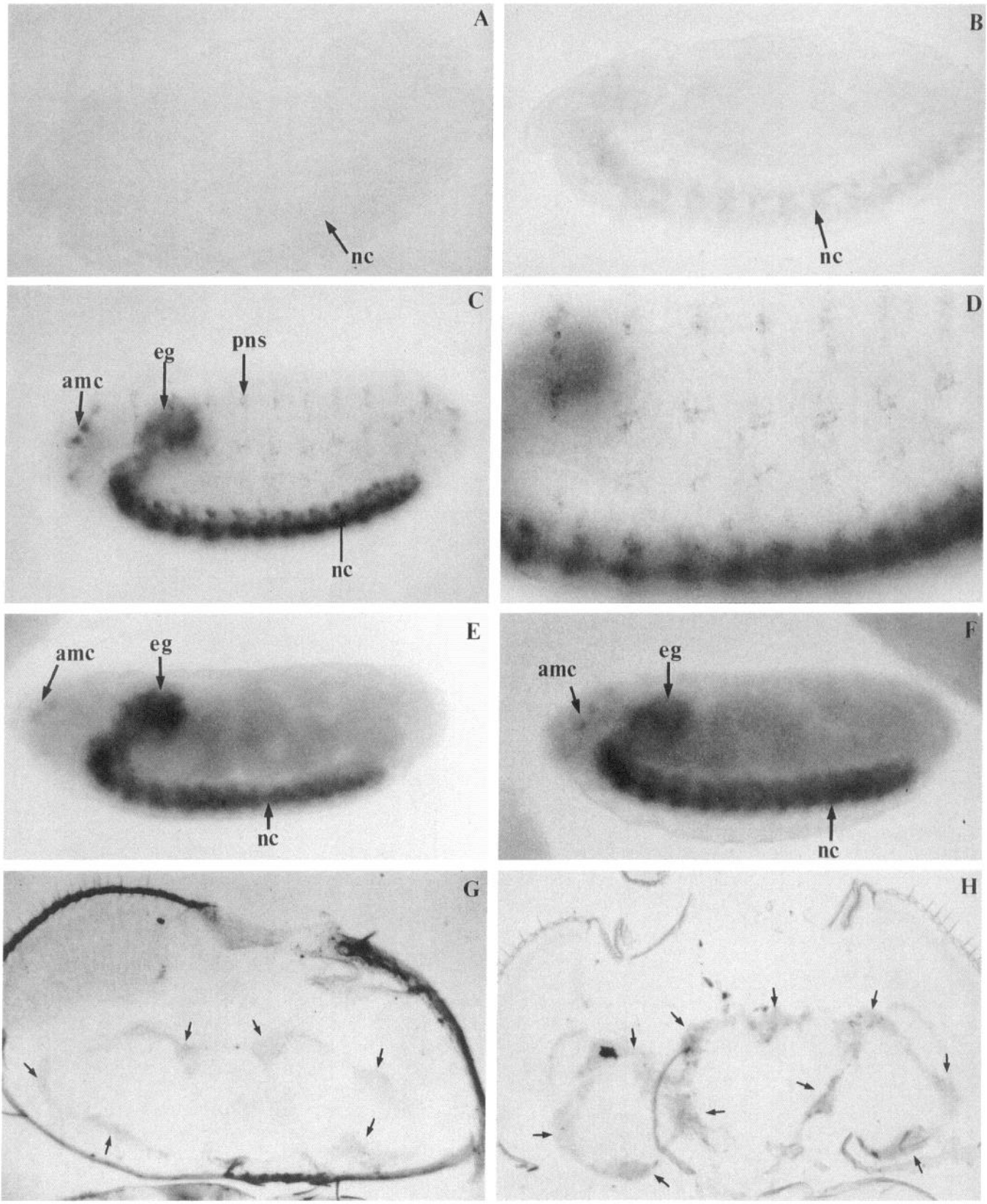

Figure 7. *In situ* hybridizations to localize transcripts in *Drosophila* whole-mount embryos and adult head sections with probes for *n-syb*, *syt*, and *drab3*. *n-syb* staining is shown in *A–D* and *G*; *syt* staining, in *F* and *H*; and *drab3* staining, in *E*. *A*, No *n-syb* staining is detected in an early whole-mount embryo (stage 11–12). *B*, The earliest detectable *n-syb* expression is apparent in a stage 13–14 embryo. *C*, A later embryo with a well-developed nervous system robustly expresses *n-syb* in both the CNS and PNS. *D*, A higher-magnification view of *C* demonstrates that *n-syb* expression is present in most and perhaps all cells of the PNS. *E*, *drab3* transcript is expressed throughout the embryonic CNS. Staining is also observed in the antennomaxillary complex of the PNS. *F*, *syt* transcript is present in the embryonic CNS and antennomaxillary complex. *G* and *H*, Sections of adult heads indicate neuronal expression of *n-syb* (*G*) and *syt* (*H*) in the cell body layers of the adult CNS (*arrows*). *amc*, antennomaxillary complex; *eg*, esophageal ganglia; *nc*, nerve cord; *pns*, peripheral nervous system.

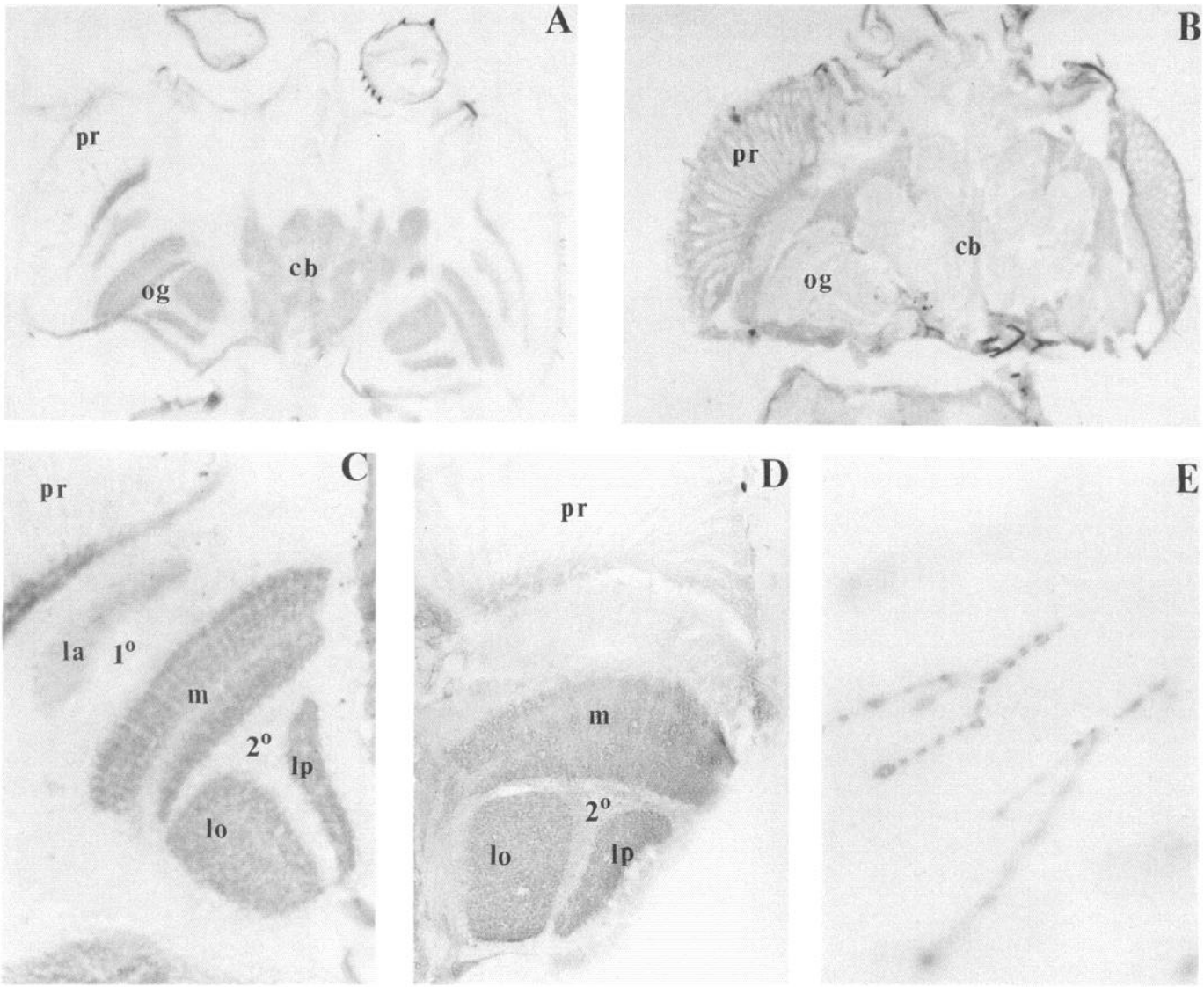

Figure 8. Immunocytochemistry of synaptotagmin and synaptobrevin. Staining with a polyclonal antibody to synaptotagmin is shown in *A–C* and *E*. Staining with a monoclonal antibody to synaptobrevin is shown in *D*. *A*, A horizontal section of an adult fly shows synaptotagmin immunoreactivity in the synaptic regions of the visual system and CNS. *B*, This section was stained and reacted in parallel to that in *A*. The staining of the synaptic regions is blocked by preincubating the polyclonal synaptotagmin antiserum with its fusion protein antigen. Background staining of the cell bodies was consistently increased by the preincubation. *C*, A higher-magnification view of the staining in the synaptic areas of the adult visual system highlights the synaptic localization of synaptotagmin. Axon tracts in the primary and secondary optic chiasms are not labeled. *D*, Anti-synaptobrevin immunoreactivity in the adult visual system is also primarily synaptic. The lamina also shows synaptobrevin immunoreactivity, although it is not revealed in the section pictured here. The synaptic regions stained in *C* and *D* correspond to the cell bodies expressing *n-syb* and *syt* described in Figure 7, *G* and *H*. *E*, Neuromuscular junctions on the body wall of whole-mount third instar larvae show synaptotagmin immunoreactivity. *cb*, central brain; *la*, lamina; *lo*, lobula; *lp*, lobular plate; *m*, medulla; *og*, optic ganglia; *pr*, photoreceptors; *1°*, primary optic chiasm; *2°*, secondary optic chiasm.

totagmin transcript was detected throughout the CNS (Fig. 7*F*) and PNS (data not shown), but not outside the nervous system. In frozen sections of adult flies, the transcript was present in the cell bodies of the CNS in both the brain (Fig. 7*H*) and the thoracic ganglia (data not shown). The transcript appeared to be ubiquitous in the cell bodies of the CNS.

Synaptotagmin *and vamp proteins are present in synaptic areas of the brain*

By screening antibodies to the vertebrate vesicle proteins for staining in *Drosophila*, we could examine the distribution of the homologs more closely and determine if the proteins were concentrated in synaptic areas. Antibodies for both synaptotagmin and synaptobrevin identified epitopes in *Drosophila* (see Materials and Methods). No suitable antibody for drab3 is yet available.

Figure 8*A* shows a frozen section of a *Drosophila* head stained with an antiserum raised against the cytoplasmic portion of rat synaptotagmin. The regions stained are the neuropil, and correspond to the synaptic connections and projections of the cell bodies that had been stained by *in situ* hybridization (Fig. 7*H*). The specificity of the synaptotagmin staining in the neuropil was demonstrated by blocking the staining by preincubating the antiserum with the fusion protein antigen (Fig. 8*B*). Figure 8*C* is a higher-magnification view of the synaptotagmin staining in the visual system. The visual system consists of a series of

integrating structures rich in synapses. The structures are the lamina, which contains most of the terminals of photoreceptor cells; the medulla, which is the next stage of visual processing; and the lobula and lobular plate, which relay the signal to the central brain and have been shown to be involved in motion detection (Buchner et al., 1984). These synapse-rich regions show significant synaptotagmin immunoreactivity, while the surrounding cell bodies do not. Several fiber tracts can be recognized in the visual system, particularly the primary and secondary optic chiasms. These axon-rich areas do not appear to contain synaptotagmin. Embryo fillets stained with synaptotagmin show a similar profile of staining in the neuropil (data not shown). In the third instar larva, the morphology of neuromuscular junctions has been characterized in detail with antibodies to neuronal surface antigens (Jan and Jan, 1982) and to glutamate (Johansen et al., 1989). We have stained for synaptotagmin in whole-mounts of first, second, and third instar larvae in which the brain and body wall musculature were exposed and pinned back. The staining at the neuromuscular junction appears tightly confined to the nerve terminals (Fig. 8*E*). The axon of the motorneuron was not stained. CNS neuropil was also darkly labeled at this larval stage.

A monoclonal antibody, Cl 10.1 (Baumert et al., 1989), raised to rat synaptobrevin was used to stain sections of adult *Drosophila.* This antibody recognizes a band of the correct molecular weight for *n-syb* on immunoblots, but also cross-reacts with one other high-molecular-weight band. Unfortunately, we cannot determine whether this cross-reactivity contributes to the cytochemical staining. Figure 8*D* shows that the staining pattern in the adult visual system resembles that of synaptotagmin. Again, the synaptic regions show greater immunoreactivity than the cell bodies or axon tracts, although it may not be as highly localized as synaptotagmin. Staining was seen in other structures in the fly, including the thoracic ganglia and abdomen; no staining above background was evident in the muscles (data not shown). Staining of embryo fillets revealed signal in the CNS and other structures including the gut. It is likely that this antibody recognizes not only the *n-syb* protein, but also the *syb* protein and possibly additional undiscovered homologs. While it is impossible to be certain that the synaptic staining is due to the *n-syb* isoform, it is suggested by the *in situ* hybridization data, which showed that *n-syb,* but not *syb,* was abundant in the nervous system. Since no additional neuronal homologs were found, and since the staining by Cl 10.1 in the nervous system was heaviest in neuropil, a synaptic localization for *n-syb* is indicated.

Discussion

We have undertaken an examination of *Drosophila* genes for synaptic vesicle proteins. With the cloning of the *n-syb* gene (Fig. 1), there are now three genes likely to encode synaptic vesicle proteins that have been identified in *Drosophila*: *n-syb, synaptotagmin,* and *drab3.*

The distributions of these three *Drosophila* proteins were examined, in part, as a prelude to a genetic analysis of the function of these proteins. The importance of determining their distribution is twofold: it provides evidence that the sequence homology is reflecting a conserved function, and it guides future efforts to identify mutations by indicating whether a widespread synaptic defect ought to be sought, or a defect at a subset of synapses. The need for such analysis became evident when the distribution of the *syb* protein was found to be more widespread than expected, suggesting that an analysis of the function of *syb* may not be relevant to synaptic vesicle physiology. We have also addressed the question of genetic redundancy by looking for additional homologs of vamp, synaptotagmin and rab3. The genetic analysis of a protein's function is complicated if the products of several genes have the same function and thus mask the phenotype of mutations in any one gene. We therefore wanted to establish whether the genes we are studying are expressed in the nervous system, where in the nervous system they are expressed, whether they are localized to synapses, and if they are the predominant isoform present in the nervous system. The expression profile and tissue distribution of each of these three genes matched that expected of a synaptic vesicle protein that was used at most, and perhaps all, synapses.

Multiple isoforms of the vamp/synaptobrevin family in Drosophila

We have cloned and sequenced a new member of the vamp/synaptobrevin family that we have called *neuronal-synaptobrevin* (*n-syb*) and mapped it to chromosome band 62A. We have demonstrated that vamp protein is present in *Drosophila* neuropil as would be expected if there were a synaptic vesicle–associated vamp. From a comparison of their expression patterns, *n-syb* is much more likely to encode this synaptic protein and to be involved in synaptic transmission than the previously identified *syb* gene (Chin et al., 1993). (1) *n-syb* expression is highly enriched in heads of flies, as would be expected for a neuronal product; *syb* is not (Fig. 4). (2) *n-syb* expression in embryos begins at about 12 hr, when synapses begin to appear (Figs. 4, 7*B*); *syb* is expressed prior to neurogenesis. (3) *In situ* hybridizations with *n-syb* probes identify transcripts specifically in neuronal tissues of embryos and adults (Fig. 7*A–D,G*); *syb* transcripts were below the level of detection in neuronal tissue but were abundant in regions of the gut and in malpighian tubules (Chin et al., 1993). Therefore, we propose that the *n-syb* gene is responsible for the production of synaptic vamp.

Staining with the monoclonal antibody Cl 10.1 was strongest in the neuropil and in the abdomen of the fly. The non-neuronal staining is probably a product of the *syb* gene or of an unidentified third vamp homolog. Although there may be additional fly genes in the *syb* family, we have found no evidence for them. In a low-stringency screen of a genomic library, in which abundance and location of expression do not influence the likelihood of recovery, we repeatedly isolated *syb* and *n-syb* clones, but not any others. In the head cDNA library, where *n-syb* was extremely abundant, no additional homolog was found. Thus, it is likely that any additional members are significantly less homologous to *syb* or to rat vamp than the two that are now known.

The presence of two vamp homologs in both the fly and the rat raises the question of whether there were two ancestral genes and whether each fly gene has a single rat equivalent. No strong relationship, however, can be discerned from comparison of their sequences. *n-syb* shows a slightly higher identity to rat vamp2 than to rat vamp1, but *n-syb* is somewhat closer to both of the rat genes than is *syb.* On a functional level, the presence of two homologs appears to serve different purposes in each species. While the two fly genes appear to have specialized for either a synaptic function or a general non-neuronal function, the two rat genes are both synaptic, though expressed in different subsets of neurons. Perhaps the nonsynaptic vamp-like proteins that have been observed on adipocyte vesicles will be a mam-

malian counterpart to the *syb* gene (Corley et al., 1992). Since the non-neuronal distribution of *syb* suggests a more general role in membrane trafficking (Chin et al., 1993), we wondered if it was particularly close to SNC1, a vamp homolog from yeast. However, *n-syb* and *syb* appear to be equidistant from the yeast SNC1. Overall similarity to SNC1 is 60% for *n-syb* and 59% for *syb*. Likewise, percentage identity to SNC1 through the conserved domains is 31% for *n-syb* and 32% for *syb*. Unlike syb, SNC1 does not have a long intravesicular carboxyl tail and its amino terminus is shorter and much less rich in prolines than any other vamp homolog. It remains unclear what motifs, if any, are important for the specifically synaptic roles of neuronal vamps. We do not at present understand why the two fly genes have exceptionally large intravesicular tails.

Does Drosophila *contain another gene coding for a synaptotagmin protein or a rab3?*

In *Drosophila*, synaptotagmin immunoreactivity appears to be present throughout the neuropil. In both *D. ommata* and rat, synaptotagmin proteins are encoded by gene families whose members are differentially expressed in regions of the nervous system. In the fly, however, the transcript for synaptotagmin is present throughout development and could provide synaptotagmin protein at all stages. Although in a previous report (Perin et al., 1991) an embryonic transcript was not observed, we have found this transcript by looking in later-stage embryos, in which the nervous system has begun to develop. In addition, the transcript is widely distributed throughout the embryonic and adult CNS. Based on the resolution of the cytology we cannot claim that every single cell expressed a particular gene, but no gaps were seen in the *in situ* hybridizations or immunocytochemistry to suggest a particular population of nonexpressing cells. Furthermore, low-stringency screening of adult head libraries with *D. ommata* p65-A, p65-C, and *Drosophila syt* provided no evidence of another synaptotagmin gene. Therefore, we suggest that the identified *synaptotagmin* gene at 23B is the predominant, if not exclusive, source of synaptotagmin protein in *Drosophila*.

In vertebrates, there is a small family of rab3 genes. Because the small GTP-binding proteins share such extensive homology, it would be very difficult to design a low-stringency screen that selectively identifies rab3 genes. Instead, we have conducted a PCR screen using degenerate rab3-specific oligonucleotides. One additional rab3 gene was identified, but it does not seem to be abundantly expressed in the adult CNS (R. W. Burgess, unpublished observations). Since *drab3* is widely expressed throughout the *Drosophila* nervous system, we suggest that this gene is the major source of rab3 protein in the fly.

The functions of n-syb, synaptotagmin, *and* drab3 *may be conserved from* Drosophila *to vertebrates*

In addition to identifying synaptic vesicle genes and addressing the question of possible genetic redundancy within gene families, we sought evidence that these proteins serve a similar function in *Drosophila* to what has been proposed in mammals, that is, a role in synaptic transmission. The structures of *n-syb, synaptotagmin,* and *drab3* are all highly conserved between flies and vertebrates. These genes are 63%, 57%, and 78% identical to their vertebrate homologs. Are they similarly localized as well? Because it has not been possible to purify intact synaptic vesicles from *Drosophila* tissue, we have examined the localization of *n-syb, synaptotagmin,* and *drab3* by *in situ* hybridization and immunocytochemistry. As we have demonstrated here, these genes also show similar expression patterns to their vertebrate cousins. All three are greatly enriched in the nervous system, apparently throughout development. For n-syb and synaptotagmin, immunolocalization of the protein to the neuropil indicates that these proteins are synaptic. Unfortunately, no antibody is presently available for localizing drab3 in flies. This conservation of both structure and localization suggests that these three proteins act at steps in neurotransmission that have been conserved from invertebrates to vertebrates.

A system for genetic analysis of vesicle proteins

If the functions of *n-syb, synaptotagmin,* and *drab3* are conserved in *Drosophila,* it should be possible to understand their function by a genetic analysis. Because each of the three genes appear to be expressed throughout the nervous system, a strong neurological phenotype would be expected for a fly deficient in one of the proteins. None of the three genes appears to be restricted to a small subset of neurons, such as the photoreceptors, which would have predicted a more limited behavioral defect, such as insensitivity to light. Nor were they restricted to a particular developmental stage, such as the adult, which would have predicted a late lethal period. The *n-syb, syt,* and *drab3* genes appear to encode the major neuronal forms of these protcins. If the proteins are essential for the function of the synapse, a deficient fly would be paralyzed and would probably die as a late-stage embryo, incapable of emerging from the egg case. A weak allele might have a more subtle neurological phenotype such as temperature-dependent paralysis, shaking, or decreased motor activity. The chromosomal location of all three genes is known: *n-syb* is at 62A, *synaptotagmin* is at 23B, and *drab3* is at 47B. No neurological mutations have yet been mapped to any of these locations. We are therefore generating mutants in each of these genes. In the case of *syt,* we have recently demonstrated that a null mutation of the gene described here is lethal. Synaptic transmission, however, persists in homozygous null first instar larvae, despite the absence of any detectable synaptotagmin protein (DiAntonio et al., 1993). Further analyses of the mutant phenotype of *syt,* as well as *n-syb* and *drab3,* may illuminate the function of these proteins in the process of neurotransmitter secretion.

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