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Expression of Synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse

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SUMMARY

Synaptotagmin is a synaptic vesicle-specific integral membrane protein that has been suggested to play a key role in synaptic vesicle docking and fusion. By monitoring Synaptotagmin's cellular and subcellular distribution during development, it is possible to study synaptic vesicle localization and transport, and synapse formation. We have initiated the study of Synaptotagmin's expression during *Drosophila* neurogenesis in order to follow synaptic vesicle movement prior to and during synapse formation, as well as to localize synaptic sites in *Drosophila*. In situ hybridizations to whole-mount embryos show that *synaptotagmin* (*syt*) message is present in the cell bodies of all peripheral nervous system neurons and many, if not all, central nervous system neurons during neurite outgrowth and synapse formation, and in mature neurons. Immunocytochemical staining with antisera specific to Synaptotagmin indicates that the protein is present at all stages of the *Drosophila* life cycle following germ band retraction. In embryos, Synaptotagmin is only transiently localized to the cell body of neurons and is transported rapidly along axons during axonogenesis. After synapse formation, Synaptotagmin accumulates in a punctate pattern at all identifiable synaptic contact sites, suggesting a general role for Synaptotagmin in synapse function. In embryos and larvae, the most intense staining is found along two broad longitudinal tracts on the dorsal side of the ventral nerve cord and the brain, and at neuromuscular junctions in the periphery. In the adult head, Synaptotagmin localizes to discrete regions of the neurophil where synapses are predicted to occur. These data indicate that synaptic vesicles are present in axons before synapse formation, and become restricted to synaptic contact sites after synapses are formed. Since a similar expression pattern of Synaptotagmin has been reported in mammals, we propose that the function of Synaptotagmin and the mechanisms governing localization of the synaptic vesicle before and after synapse formation are conserved in invertebrate and vertebrate species. The ability to mark synapses in *Drosophila* should facilitate the study of synapse formation and function, providing a new tool to dissect the molecular mechanisms underlying these processes.

Key words: synapse, synaptic vesicle, neurogenesis, fruitfly, neuromuscular junction, *Drosophila*, Synaptotagmin

INTRODUCTION

Neurogenesis in both vertebrate and invertebrate species requires a complex maturation of groups of neurons that form highly organized synaptic contacts with a variety of target tissues. Communication at these synapses involves release of neurotransmitters from synaptic vesicles that fuse with the presynaptic membrane following the influx of extracellular calcium. Synaptic vesicle proteins are then retrieved from the presynaptic membrane and recycled into synaptic vesicles for further rounds of neurotransmitter release (for review, see Sudhof and Jahn, 1991). The biochemical events underlying neurotransmitter release and the trafficking of synaptic vesicles may be similar in both vertebrate and invertebrate synapses, since electrophysiological data and morphological analyses at the light and electron microscopy level indicate conserved functional and structural properties of the synapse (Jan and Jan, 1976; Budnik et al., 1990). Hence, proteins of the synaptic vesicle that are conserved throughout evolution are likely to play similar functions in distantly related organisms. One such family of proteins are the Synaptotagmins, integral membrane proteins of synaptic vesicles, which exhibit 57% overall identity between rat (when discussing activities and homology we will be referring to rat Synaptotagmin I) and *Drosophila* (Perin et al., 1991a). Synaptotagmin has been identified as part of a multi-gene family in several vertebrate species (Geppert et al., 1991; Wendland et al., 1991). All isoforms contain a single transmembrane region and a cytoplasmic carboxy-terminal moiety with two repeats homologous to a domain known to be involved in calcium-dependent membrane interactions (Perin et al., 1990). In

addition, Synaptotagmin has been shown to bind acidic phospholipids in a calcium-dependent manner and to mediate the bridging of membranes (Perin et al., 1990; Brose et al., 1992). Synaptotagmin has also been reported to interact with ω-conotoxin-sensitive calcium channels (Leveque et al., 1992), the presynaptic receptor for latrotoxin (Petrenko et al., 1991), and another presynaptic membrane protein, syntaxin (Bennett et al., 1992). These data implicate Synaptotagmin both in docking synaptic vesicles to their presynaptic release sites, and in the calcium-dependent fusion process.

We have chosen to investigate Synaptotagmin's role in neurotransmission in vivo and have therefore initiated this study in *Drosophila.* The expression pattern of Synaptotagmin in embryos may demonstrate the distribution of synaptic vesicles before and after synaptogenesis and the timing and location of synapse formation. Several general neuronal markers have been used to identify neurons and their processes in *Drosophila*. Using antibodies against horse radish peroxidase and glutamate, synaptic sites in the bodywall musculature of embryos (Johansen et al., 1989b) and larvae (Johansen et al., 1989a; Budnik et al., 1989, 1990) have been identified. In addition, neuronal dye fills and electron microscopy have identified some neuronal terminations in embryos (Sink and Whittington, 1991), larvae (Johansen et al., 1989a) and adults (Koenig et al., 1989; Costello and Wyman, 1986). However, relatively little is known about the location of many synapses, particularly those outside the neuromuscular junction and primary sensory centers in the adult brain. In particular, it is unclear when and where synapses form between neurons in the CNS of the embryo. Moreover, it is unclear in *Drosophila* whether synaptic vesicles are only made and transported after synapse formation, or whether synaptic vesicles are also made during axonogenesis and can subsequently localize to synaptic contact sites. In addition, the lack of an easily identifiable synaptic marker in invertebrates has slowed the identification of proteins that are involved in synaptic targeting and synapse formation.

We detect the presence of only one *Drosophila synaptotagmin (syt)* gene that is transcribed throughout development and is expressed by most, if not all, neurons. Based on the developmental expression pattern of Synaptotagmin during neurite outgrowth and synaptogenesis, we conclude that synaptic vesicles or their precursors are made and transported along the axon prior to synapse formation. Once synaptic contacts have been established, synaptic vesicles localize to these sites within a brief time period. Synaptotagmin immunocytochemistry also shows that most synapses are highly concentrated in the CNS of embryos. These antisera are the first to label most if not all synapses in *Drosophila*, and should be of general use to study synapse formation and function.

MATERIALS AND METHODS

cDNA cloning and sequencing

A 9- to 12-hour-old embryonic cDNA library (Zinn et al., 1988) was screened with ^{32}P-labeled oligos and random-primed labeled cDNA fragments derived from the previously identified *Drosophila syt* cDNA isolated from adult flies (Perin et al., 1991a). Library filters were hybridized overnight at 42°C in 25% formamide. Filters were washed with 6× SSC, 0.1% SDS (oligos) or 2× SSC, 1% SDS (labeled fragments) twice at 50°C for 50 minutes. Sequencing of the positive cDNAs was performed using the dideoxy nucleotide chain termination method (Sanger et al., 1977).

RNA blotting

Poly(A)+ RNA was prepared from developmental stages of Canton-S flies. 5 μg of RNA was electrophoresed in formaldehyde gels and blotted to nylon. Blots were probed with uniformly ^{32}P-labeled cDNA probes corresponding to the entire coding region or smaller fragments of *Drosophila syt* (Perin et al., 1991a). Blots were then washed at high stringency (0.1× SSC, 0.1% SDS) and exposed for one week at −70°C with intensifying screens.

Generation of Synaptotagmin antibodies

Two rabbit polyclonal antibodies were raised against *Drosophila* Synaptotagmin. Antibody DSYT1 was generated against a synthetic peptide, corresponding to the 15 amino-terminal amino acids of Synaptotagmin, coupled to keyhole limpet hemocyanin. Antibody DSYT2 was prepared against a bacterial produced protein containing residues 134-474, which corresponds to the complete cytoplasmic sequence following the transmembrane region. The production of bacterial recombinant proteins was as described by Perin et al. (1991b). After induction, the bacterial pellet was resuspended, run on a preparative SDS-PAGE gel, and the appropriate band excised. 100 μg of protein was injected 5 to 6 times subcutaneously in New Zealand white rabbits before bleeding. The production of RSYT1 (prepared against rat recombinant Synaptotagmin) and RSYT2 (prepared against a peptide containing the 12 amino-terminal residues of rat Synaptotagmin) has been previously described (Perin et al., 1991b). Polyclonal antisera RSYT3 was generated against the carboxy-terminal 34 amino acids of rat Synaptotagmin 1.

In situ hybridization and immunocytochemical staining

Whole-mount in situ hybridizations were performed as described by Tautz and Pfeifle (1989). Immunocytochemical staining of embryos was carried out as described by Bellen et al. (1992). Antibody staining of third instar larvae was as described by Johansen et al. (1989a). Immunocytochemical staining of adult heads was performed as described by Han et al. (1992). Primary and secondary antibodies were preabsorbed to 0- to 9-hour-old embryos overnight at 4°C. DSYT2 was used at a final concentration of 1:500 in embryos and larvae, and 1:1000 in adult head sections. DSYT2 staining is highly reproducible under these conditions; however, staining in the embryonic PNS is restricted after cuticle formation (after stage 17) due to poor penetration of the antibodies. This limitation can be readily overcome by disrupting the cuticle of late embryos, thus providing greater access for the antibodies. Monoclonal antibody (mAb) BP104 (Hortsch et al., 1990) was used at 1:10, and mAb. 22 C10 (Zipursky et al., 1985) at 1:10. Secondary anti-mouse and anti-rabbit antibodies were used at 1:200.

Immunoblots and trypsin digestion

Crude synaptic vesicle preparations were made by freezing 10,000-20,000 flies in liquid N_2 and separating heads from bodies using a sieve. Frozen heads were crushed in liquid N_2 and resuspended in 0.32 M sucrose with addition of protease inhibitors (5 μg/ml Leupeptin, 5 μg/ml Pepstatin, 5 mM Benzamidine, 0.25 mM PMSF, 10 mM EDTA, 1 mM EGTA; final concentrations) and homogenized. A 20 minute, 15,000 ***g*** spin was used to pellet cell debris and cuticle. A fraction enriched in synaptic vesicles was pelletted with a 1 hour 150,000 ***g*** spin. A similar procedure with

mammalian brain tissue yields 70-80% pure synaptic vesicles. SDS-PAGE and immunoblotting were performed as described by Perin et al. (1988). Abs. DSYT1 and DSYT2 were used at 1:200 on westerns. Antibody-reactive bands were visualized with peroxidase-labeled secondary antibodies and enhanced chemiluminescence detection (Amersham). Synaptic vesicles (100 μg of protein) were digested with 10 ng trypsin in 100 mM NaCl, 20 mM Hepes-NaOH for 1 hour at 37°C. Reactions were stopped by boiling in electrophoresis sample buffer (62 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 2% SDS).

Fly stocks

Flies were raised at 24°C on standard medium with addition of baker's yeast. All flies used in this work are Canton-S.

RESULTS

Synaptotagmin is transcribed exclusively in neurons

Two to three different *syt* genes have been identified in several vertebrate species (Geppert et al., 1991; Wendland et al., 1991). To determine whether different genes encoding Synaptotagmin-like proteins exist in *Drosophila* and to determine whether adult *syt* transcripts encode the same protein as in embryos, we have carried out low-stringency cDNA screens, genomic southern analysis and in situ hybridizations to embryos and polytene chromosomes. We have screened a 9-12 hour embryonic cDNA library (Zinn et al., 1988) at low stringency with the previously characterized *syt* cDNA from adult flies (Perin et al., 1991a). Eleven positive plaques were picked at random, cloned, restriction enzyme mapped and partly sequenced. All embryonic clones examined have similar restriction enzyme maps and identical sequences over large regions of the open reading frame (ORF) when compared to those of the adult *syt* cDNA (data not shown). In addition, genomic Southern blots using the embryonic and adult cDNAs as probes reveal identical bands (data not shown). The cDNAs of both adults and embryos hybridize to cytological bands 23A6-23B1 of third instar larvae polytene chromosomes. These observations suggest that all isolated cDNAs are derived from a single locus. We have also failed to identify additional Synaptotagmin isoforms with western analysis (see below). These observations suggest that a single *syt* gene is present in *Drosophila* and that the expression pattern described below is specific for *syt*.

To determine the complexity and size of *syt* transcripts, we performed a developmental northern analysis. As shown in Fig. 1, *syt* transcripts can be detected at all stages of development after germ band retraction. Expression of a 4.5 kb *syt* message appears between 8 and 12 hours of embryogenesis and is present at all subsequent developmental stages. A 7.0 kb message appears in 12-20 hour embyros and is present throughout development. An approximately 1.5 kb transcript is seen in third instar larvae and pupae. We have previously reported the presence of adult transcripts of 7 and 4.5 kb (Perin et al., 1991a). To determine the relationship of these transcripts to the previously published *syt* cDNA (Perin et al., 1991a), northern blots were probed with different fragments from the cDNA. Probes derived from the 5′ region of the cDNA, including both coding (bps 435-795) and noncoding sequences (bps 0-270, 0-430), identify all three transcripts. However, probes derived from the 3′ untranslated region (bps 1600-1900), as well as the 3′ coding region (1220-1900), only hybridize to the 4.5 and 7 kb messages, indicating that the 1.5 kb message may represent a transcript generated by alternative splicing or premature termination. This transcript may thus only contain approximately half of the ORF and is possibly nonfunctional. The 4.5 and 7 kb transcripts are recognized by small fragments spanning the entire coding region, suggesting that these messages may represent transcripts that differ in their untranslated region. These data indicate that *syt* is transcribed at most stages of development, including stages during which active synaptogenesis and neuronal modeling occur.

To study the tissue distribution of *syt* in embryos, whole-mount in situ hybridization experiments were performed using digoxigenin-labeled *syt* cDNA probes. As shown in Fig. 2, *syt* is first expressed in the CNS in a few neurons in each segment of the ventral nerve cord (VNC) in late stage 13 embryos. During stage 14, the pattern in the VNC becomes more complex, and the cell bodies of more neurons are labeled. At stage 15, intense staining can be found in the supra- and suboesophageal ganglia, as well as in the VNC. In addition, a subset of cells of the PNS are stained. As shown in the schematic drawing and PNS in situ inset of Fig. 2E, all the cells of the PNS that stain correspond to neurons. Individual neurons from the lateral PNS cluster are labeled in Fig. 2E, demonstrating that none of the support cells including glia are labeled. Although it is difficult to determine if all neurons in the CNS express *syt*, many

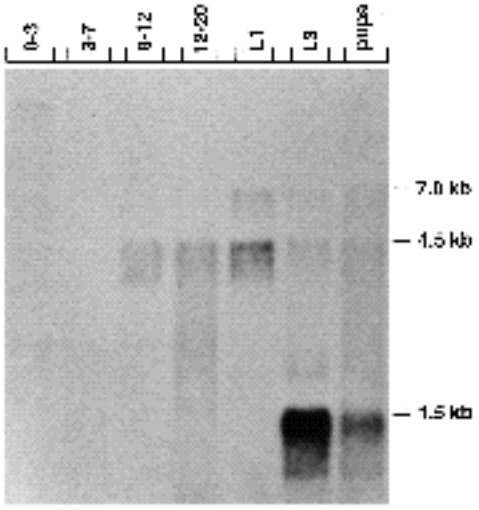
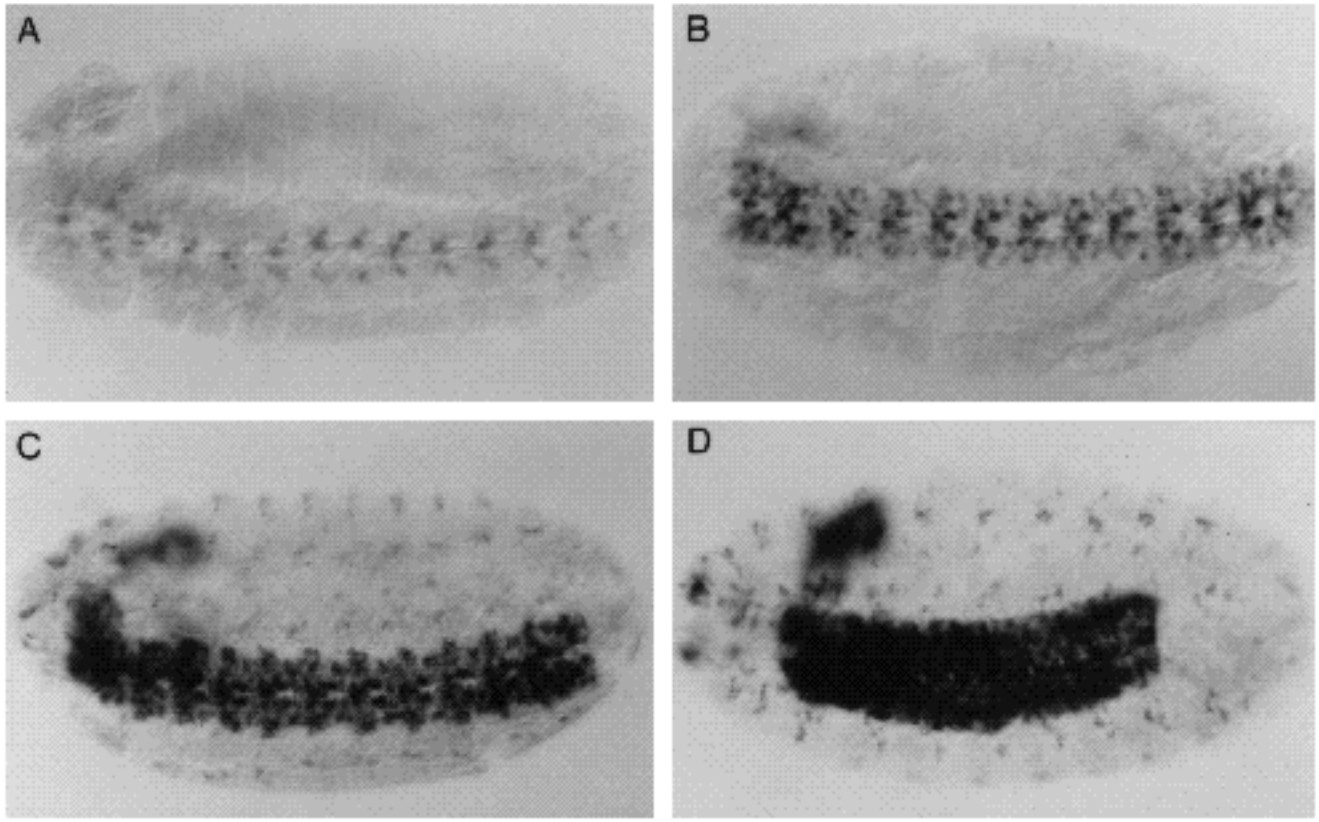


Fig. 1. Developmental Northern using *syt* cDNA as a probe. *syt* messages first appear in 8-12 hour embryos and are present during all stages of development. Three transcripts of approximately 7.0, 4.5 and 1.5 kb can be detected. The 4.5 kb message appears in 8-12 hour embryos and is present throughout the life cycle. The 7 kb message appears in 12-20 hour embryos and is present at all other stages of development. The 1.5 kb message is only present in third instar larvae and pupae and may represent an alternative spliced product or premature termination that results in a message that lacks significant portions of the ORF. These messages all seem to be derived from a single *syt* gene (see text).

neurons in the VNC and brain are stained. During embryogenesis, we observed no *syt* expression in non-neuronal cells. We conclude that *syt* is a neuronal-specific marker, which is expressed in all PNS and many, if not all, CNS neurons during the final embryonic differentiation stages and in the fully developed embryonic nervous system.

Polyclonal antisera against Synaptotagmin recognize a 69×10^3 M_r protein of synaptic vesicles

To determine the cellular and subcellular distribution of Synaptotagmin during embryonic development, we generated polyclonal antibodies against a peptide containing the 15 amino-terminal amino acids of Synaptotagmin



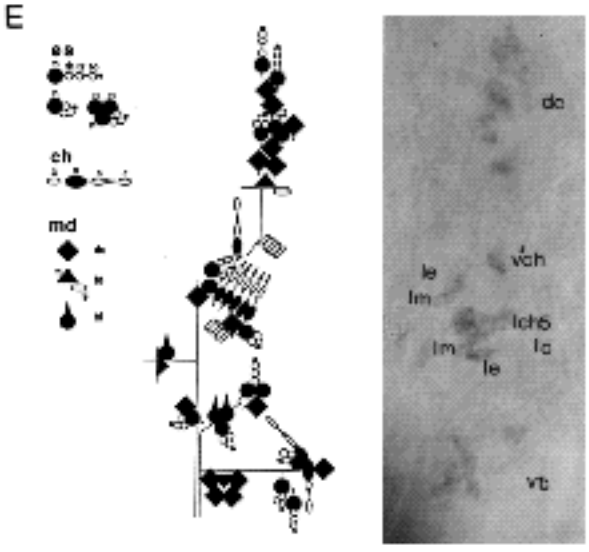


Fig. 2. Embryonic whole-mount in situ hybridizations using a digoxigenin-labeled *syt* cDNA. *syt* is expressed in the later phases of the differentiating embryonic nervous system. Embryos are shown anterior to the left, dorsal up. All views are ventrolateral views. Staging is as described by Campos-Ortega and Hartenstein (1985). (A) Stage 13-14 embryo. Note that specific cells along the midline of each segment of the ventral nerve cord (VNC) are stained. (B) Stage 15 embryo. Many more cells in the VNC and brain express *syt* in a segmentally repeated pattern. (C) Stage 16 embryo. There is intense staining in the supra- and suboesophageal ganglion as well as in the VNC. PNS neurons also express *syt* weakly. (D) Stage 17 embryo. *syt* is expressed abundantly in the CNS and PNS. E. A schematic diagram (redrawn from Bodmer et al. (1989)) in which all cells that express *syt* in the PNS are labeled in black. The photograph shows an abdominal segment of a *Drosophila* embryo from our in situ hybridizations with focus on the lateral cluster (lc). Cells that express *syt* correspond to PNS neurons. The support cells of the PNS do not express *syt*. Individual neurons of the dorsal cluster (dc) and ventral cluster (vc) can also be individually identified, but are not in focus in this photograph. Only the v′ cluster is shown in the photograph. Abbreviations: es, external sensory organ; ch, chordotonal organ; md, multiple dendrite neurons; n, neuron; th, thecogen cell; tr, trichogen cell; to, tormogen cell; li, ligament cell; s, scolopale cell; c, cap cell; da, dendritic arbor neuron; bd, bipolar dendrite neuron; g, glial cell; td, trachea innervating cell; le, lateral external sensory neuron; lm, lateral multiple dendrite neuron; v′ch, v′ chordotonal neuron; lch5, the five lateral chordotonal neurons. From dorsal to ventral: the dorsal cluster, the lateral cluster, and the two ventral clusters

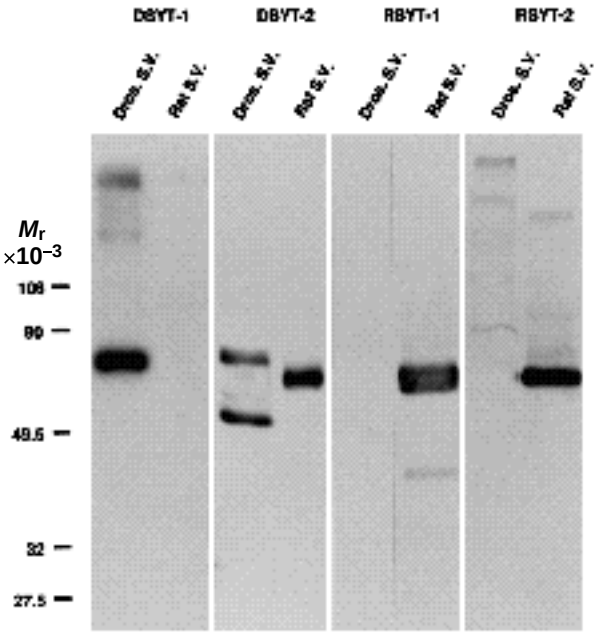


Fig. 3. Immunoblots of partially purified synaptic vesicles of *Drosophila* and rat using antibodies against the amino-terminal amino acids, DSYT1 and RSYT2, and the remainder of the protein, DSYT2 and RSYT1, are shown. The antibodies recognize a 69×10^3 M_r protein in *Drosophila* and a 65×10^3 M_r protein in rat. Note that the DSYT2 antibody recognizes Synaptotagmin of both species. DSYT2 consistently recognizes two protein bands in *Drosophila* synaptic vesicles (see Fig. 4).

coupled to keyhole limpet hemocyanin (Ab. DSYT1). In addition, a bacterial recombinant protein containing the cytoplasmic portion of *Drosophila* Synaptotagmin (Perin et al., 1991a) was prepared and used as an immunogen (Ab. DSYT2). Polyclonal antibodies against similar domains of the rat protein (Ab. RSYT1 against recombinant rat Synaptotagmin and Ab. RSYT2 against an amino-terminal peptide) were also used in order to compare the *Drosophila* and rat proteins. As shown in Fig. 3, Ab. DSYT1 recognizes a 69×10^3 M_r protein from *Drosophila* synaptic vesicles, but fails to recognize proteins of rat synaptic vesicles. This is expected because the amino-terminal domain of Synaptotagmin is highly divergent between rat and *Drosophila*. Ab. DSYT2 recognizes a protein of the same molecular weight as DSYT1 in *Drosophila* synaptic vesicles, as well as rat Synaptotagmin (see Fig. 3). Ab. DSYT2 also recognizes a protein of 55×10^3 M_r in *Drosophila* synaptic vesicles, which probably represents a breakdown product of Synaptotagmin (see below). Abs. RSYT1 and RSYT2 recognize a 65×10^3 M_r protein in rat synaptic vesicles. Ab. RSYT1 occasionally shows faint reactivity with a 69×10^3 M_r protein of *Drosophila* synaptic vesicles. These results indicate that antisera to Synaptotagmin from *Drosophila* and rat are specific for Synaptotagmin.

Since Ab. DSYT2 recognizes two bands in synaptic vesicle preparations, we further defined the specificity of the antisera. Trypsin digestion of *Drosophila* synaptic vesicles was performed in order to examine the appearance of breakdown products of Synaptotagmin. As shown in Fig. 4A, Ab. DSYT1 recognizes a major amino-terminal trypsin fragment of approximately 31×10^3 M_r, while Ab. DSYT2 recognizes a carboxy-terminal fragment of approximately 38×10^3 M_r in *Drosophila* synaptic vesicles. Both Ab. DSYT2 and Ab. RSYT1 recognize the same 36×10^3 M_r breakdown product in rat synaptic vesicles, indicating that the DSYT2 antibody recognizes a conserved region of the cytoplasmic domain of Synaptotagmin (see Fig. 4B). The presence of a single hypersensitive trypsin site in rat Synaptotagmin (as shown in Fig. 4B) has been previously reported (Perin et al., 1991a). We find the presence of a hypersensitive site in a similar location in *Drosophila* Synaptotagmin. The additional band at 55×10^3 M_r recognized by Ab. DSYT2, but not by Ab. DSYT1, appears to be an alternate step in the breakdown of Synaptotagmin. Indeed, trypsin digestion eliminates the 55×10^3 M_r band with a concomitant increase in the 38×10^3 M_r product and without production

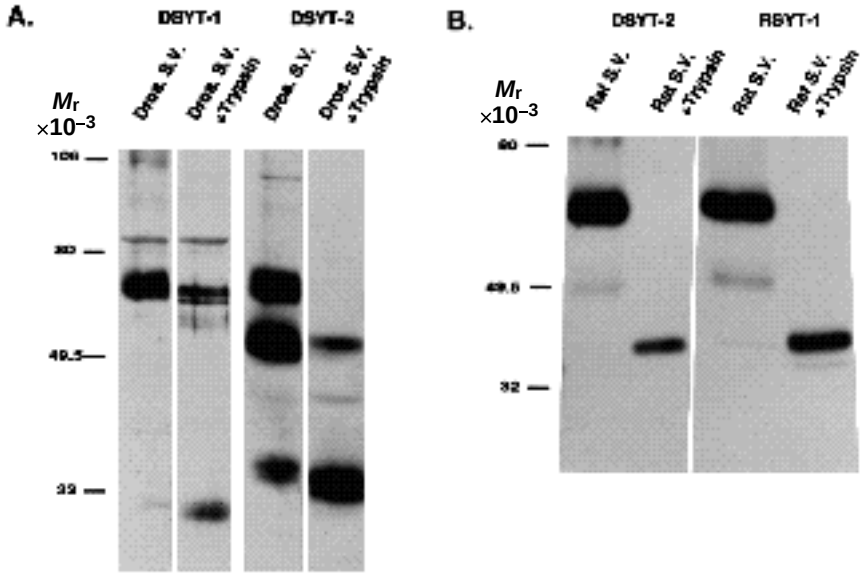


Fig. 4. Western analysis of synaptic vesicle preparations digested with trypsin. The DSYT1 and DSYT2 antibodies recognize independent domains of Synaptotagmin. **(**A) Upon trypsin digestion of synaptic vesicles DSYT1 identifies a amino-terminal 31×10^3 M_r moiety, whereas DSYT2 recognizes a 38×10^3 M_r carboxy-terminal fragment, as well as a 55×10^3 M_r protein that is probably an intermediate in the breakdown process (see text). (B) The DSYT2 and RSYT1 antibodies both recognize the same 36×10^3 M_r trypsin breakdown product in rat synaptic vesicles, providing additional evidence that these antibodies specifically recognize Synaptotagmin.

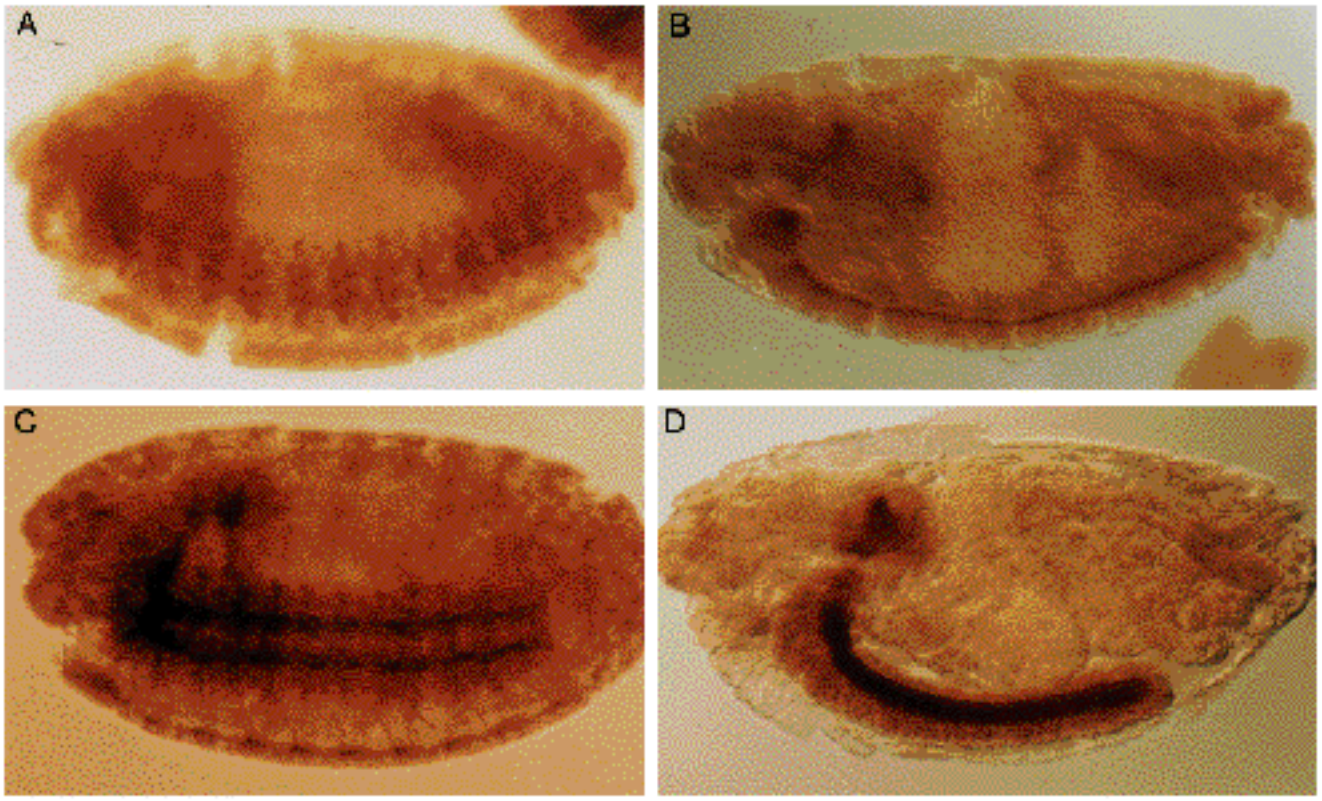


Fig. 5. Immunocytochemical staining of whole-mount embryos using the DSYT2 antibody. Synaptotagmin is rapidly transported to the synapses and localizes synaptic contact sites. Anterior is to the left, dorsal is up. (A) Ventrolateral view. Stage 14 embryo. Note that many cell bodies of each segment of the ventral nerve cord (VNC) are stained. The cell bodies of the neurons are only intensely labeled for a very short period. (B) Stage 15 embryo. Lateral view, focal plane is on the center of the embryo. Note that most protein has accumulated in the dorsal part of the VNC and the brain. (C) Stage 16 embryo. Ventrolateral view. Synaptotagmin is mostly localized along the longitudinal tracts but is also present in many other areas of the CNS (see Fig. 6B). Staining can also be observed transiently in the axons of the motorneurons and the PNS neurons during this stage. The commissures of the VNC stain only weakly. (D) Stage 17 embryo. Lateral view, focal plane is on the CNS of the embryo. Synaptotagmin is mainly localized along the two longitudinal tracts of the CNS. Synaptotagmin is also present at synapses in the bodywall musculature at this stage (not in focal plane). Ab. DSYT2 also shows faint reactivity with an antigen present at low levels in a subcellular compartment of the pole cells (not shown). These cells, however, lack *syt* message. No other non-neuronal cells are labeled by Ab. DSYT2.

of other trypsin products. This 55×10^3 M_r protein lacks the amino-terminal epitope recognized by Ab. DSYT1 and probably represents a breakdown product lacking the terminal portion of the luminal domain. Additional evidence indicating that the 55×10^3 M_r protein is indeed Synaptotagmin, and not a cross-reacting protein, was obtained from immunocytochemical staining of embryos with a small deficiency (Df (2L) C144; 23A1-23C; Jeff Sekalsky and Bill Gelbart, personal communication) that uncovers the *syt* locus. Embryos homozygous for this deficiency showed no staining with DSYT2, suggesting that the protein is encoded by a gene contained within this deficiency, and hence most likely corresponds to Synaptotagmin. To confirm this hypothesis, an antibody against the carboxy-terminal 34 amino acids of rat Synaptotagmin I, a region highly conserved between rat and *Drosophila*, was prepared and tested on synaptic vesicle preparations. This antisera (RSYT3) also recognized proteins of 69×10^3 and 55×10^3 M_r in *Drosophila* synaptic vesicles, as well as a protein of 65×10^3 M_r in rat synaptic vesicles (data not shown). Although we believe this product to be an alternate step in the degradation of Synaptotagmin, we cannot completely rule out that the 55×10^3 M_r protein is a splicing variant of Synaptotagmin.

To determine whether Synaptotagmin is present throughout the life cycle of *Drosophila*, we prepared crude synaptic vesicle extracts from 0-20 hour embryos, first and third instar larvae, pupae and adults. Western analysis with Abs. DSYT1 and DSYT2 shows that a 69×10^3 M_r protein and the above described breakdown products are present at all stages of development (data not shown). This observation, combined with the previous sequencing and in situ hybridization data, suggests that Synaptotagmin is present as a single isoform at all stages of development. In addition, no other bands are detected in western blots of whole extracts, indicating that the antisera do not recognize additional proteins from *Drosophila*.

The sequence of the *syt* ORF predicts a protein of 50×10^3 M_r in *Drosophila* and 48×10^3 M_r in rat. Part of the difference between observed and predicted M_r in the rat protein has been shown to be due to glycosylation of the mammalian form (Perin et al., 1991b). Endoglycosylase F digestion of

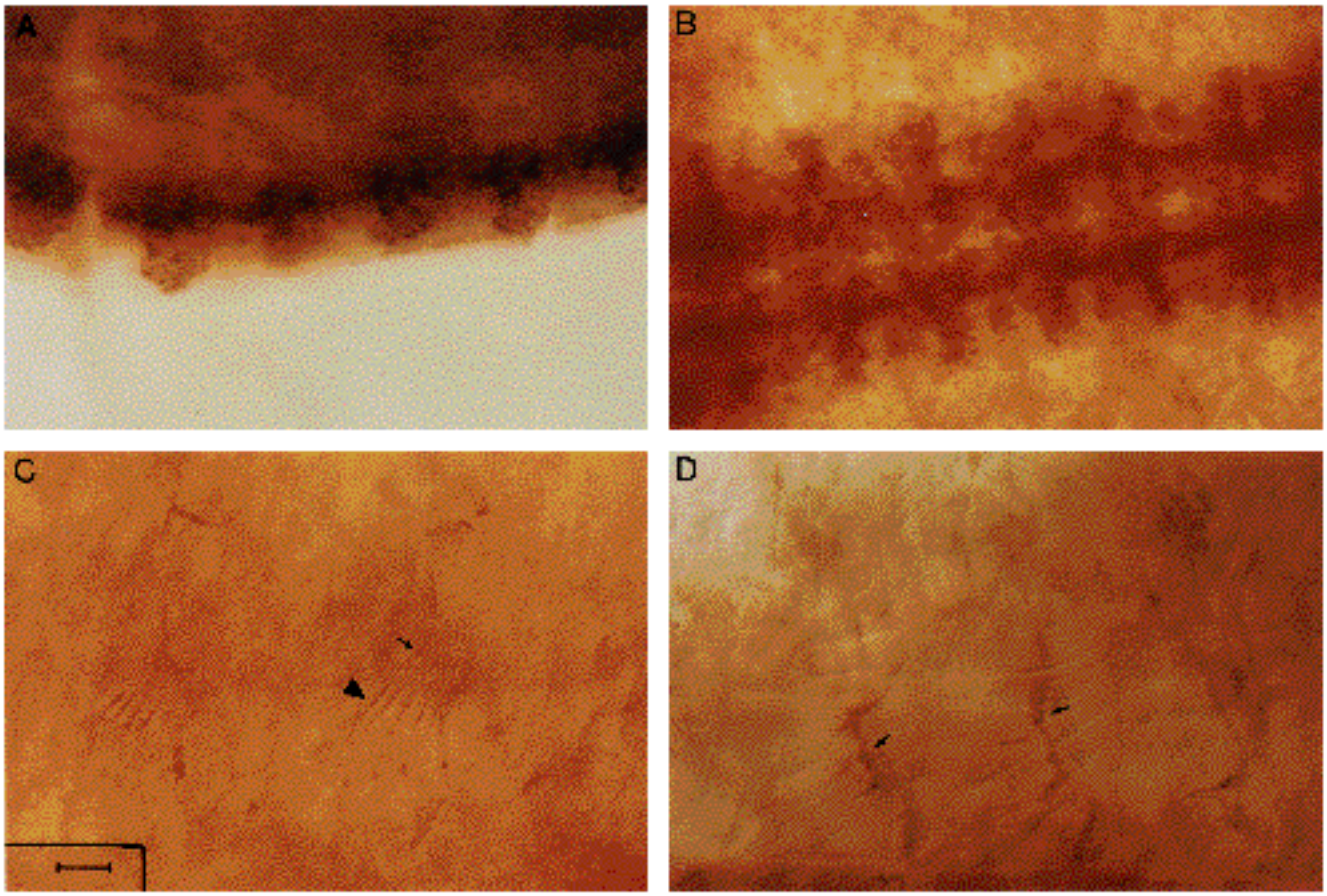


Fig. 6. Immunocytochemical staining of whole-mount embryos using Ab. DSYT2. Subcellular localization of Synaptotagmin during embryonic development. (A) Detailed view of the VNC of the CNS of a stage 14 embryo. Note the clusters of neurons in each segment that express Synaptotagmin. Synaptotagmin is mostly localized to a subcellular compartment associated with the membrane, possibly the Golgi complex. (B) Detailed view of the CNS of a stage 16 embryo. Most staining accumulates along the longitudinal tracts and at the sites of incoming peripheral neurons. (C) Detail of the chordotonal organs of the lateral cluster of the PNS of a stage 16 embryo (see also Fig. 1 E). Note that Synaptotagmin is transiently present in the dendrites of the chordotonal neurons (arrow head) and in the scolopales (arrow). (D) Detail of the ventral region of a stage 17 embryo with focus on the surface of the ventral muscles. The tape-like structures are muscles. The arrowheads point to multiple synapses of neuromuscular junctions of two segments.

Drosophila synaptic vesicles did not cause a M_r shift of the protein on SDS-PAGE gels (data not shown), indicating that Synaptotagmin may not be N-linked glycosylated in *Drosophila*. However, when the cytoplasmic domain of Synaptotagmin is expressed in *E. coli*, a recombinant protein of 41×10^3 M_r is made, only slightly larger than the predicted molecular mass of 39×10^3 (Perin et al., 1991a). Taken together, these observations suggest that other secondary modifications or secondary structure of the full-length protein may account for the molecular weight differences that we observe.

Synaptotagmin expression reveals trafficking of synaptic vesicles and localizes sites of synaptic contact

Ab. DSYT2 was used for immunocytochemical staining of whole-mount embryos using a secondary antibody coupled to horseradish peroxidase. A developmental profile of the expression pattern of Synaptotagmin is shown in Fig. 5. This expression pattern correlates well with the cellular distribution of *syt* seen in whole-mount in situ hybridization experiments, yet reveals a very different subcellular distribution of the protein compared to the message. Expression of Synaptotagmin is detected at low levels in late stage 13 or early stage 14 embryos in a subset of neurons of the CNS (see Fig. 5A). As shown in Fig. 6A, Synaptotagmin is localized to the cell body of clusters of neurons in the ventral part of the VNC. Some punctuate or granular staining can be seen in these neurons close to the cell membrane (see Fig. 6A). Although it is difficult to determine the precise subcellular localization at the light microscope resolution, this staining may correspond to the Golgi apparatus. At later stages of embryonic development, very little staining can be observed in the cell bodies of neurons, except transiently in the neurons of the PNS. During stage 15 and later, most Synaptotagmin accumulates along the dorsal longitudinal tracts of the VNC and brain (Fig. 5B-D). The commissures in the VNC stain only weakly (Fig. 5C). As shown in Fig. 6B, staining in the CNS is not only confined to the area along the longitudinal tracts. Punctate clusters of staining can be

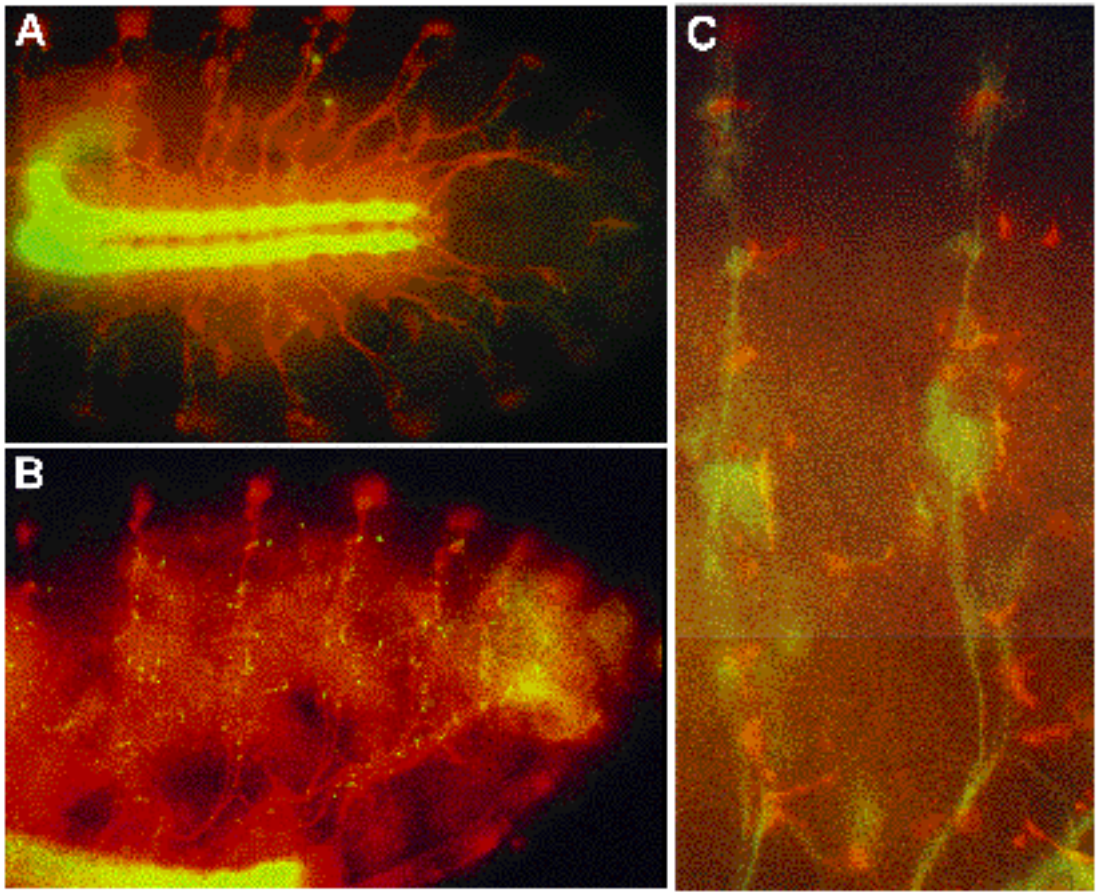


Fig. 7. Immunofluorescent staining of whole-mount embryos with Ab. DSYT2 and MAb. 22C10. Synaptotagmin is present in a segmentally repeated pattern at neuromuscular junctions and identifies sites of synaptic contact in the mature embryo. Anterior is to the left, dorsal is up. (A) Immunocytochemical staining of a whole-mount stage 17 embryo with MAb. 22C10 (red) and polyclonal Ab. DSYT2 (green). Areas that are double labeled stain yellow. (B) Lateral view of the posterior abdominal segments of a stage 17 embryo stained as in A. Synaptotagmin accumulates in the synapses at the neuromuscular junctions (green dots). (C) Detail of two abdominal segments of a stage 17 embryo labeled with MAb. 22C10 which labels the axons (green) and DSYT2 which labels the neuromuscular junctions (red). Note the stereotyped patterns of the synapses with the muscles, as well as the diversity of synaptic shapes within a single segment.

seen lateral to the longitudinal tracts, and larger and smaller clusters alternate at the positions were the intersegmental and segmental nerves connect to the CNS. Thus, we conclude that most synapses in the CNS are located along the longitudinal tracts of the VNC and brain, and that they are non-randomly distributed.

In the periphery, expression of Synaptotagmin can be observed transiently in the afferent neurons of the sensory PNS. Expression of Synaptotagmin in these neurons is weak. During stages 15 and 16, the axons of PNS neurons also label transiently (Fig. 5C), suggesting that synaptic vesicles or their precursors are present in axons prior to synapse formation. As shown in Fig. 6C, expression of Synaptotagmin is not only restricted to axons, since the dendrites of the chordotonal neurons and their scolopales also contain Synaptotagmin transiently. In stage 17 embryos, staining in the periphery is confined to a punctate staining pattern at neuromuscular junctions. As shown in Fig. 6D, expression at these synapses is quite abundant. Double-labeling studies using immunofluoresence with MAb 22C10 (a marker for neuronal cell membranes) or MAb BP104 (a marker for neuroglian, a cell adhesion molecule) and Ab. DSYT2 indicate that all neuromuscular junctions in a particular abdominal segment express Synaptotagmin. As shown in Fig. 7A and B, the pattern of synaptic connections within a segment is highly organized and conserved throughout the segments. However, embryonic neuromuscular junctions within each segment show a variety of shapes and structures (see Fig. 7C). Some are delta-like, others are crescent shaped, and still others are straight. These synaptic contact sites increase in button number and distribution as development proceeds through the larval stages. Synaptic vesicle clustering at neuromuscular junctions of the *Drosophila* embryo occurs essentially simultaneously throughout each segment during stage 16. The synaptic organization of the body wall musculature innervated by the segmental and intersegmental nerve, as revealed by Synaptotagmin localization, is in agreement with that previously described by Johansen et al. (1989b).

Synaptotagmin is also expressed at synaptic contact sites

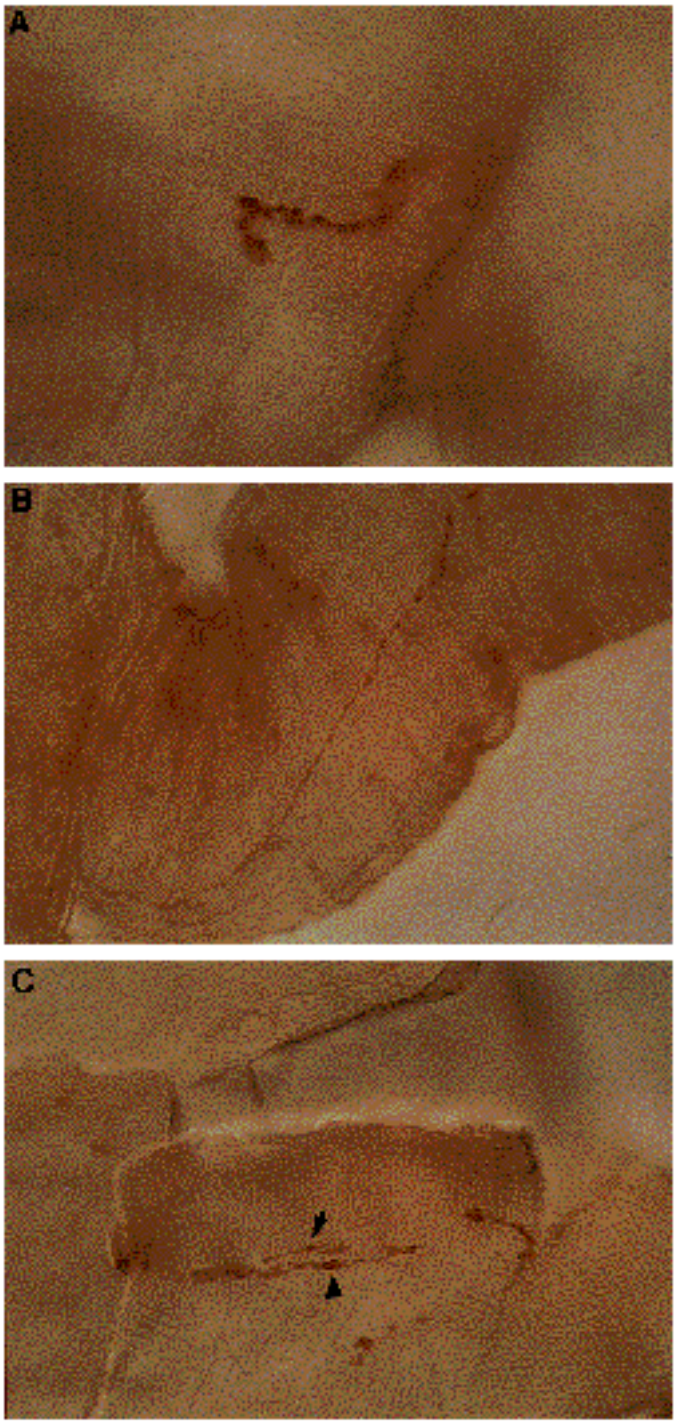


Fig. 8. Immunocytochemical staining of the ventral musculature of a third instar larvae with Ab. DSYT2 using a secondary antibody coupled to horse radish peroxidase. (A) Labeling of a third instar larval neuron with Ab. DSYT2. Note the very large synaptic terminals along the muscle fiber and the proximity of the terminals to the site of innervation. This staining is characteristic of type I processes. (B) DSYT2 staining in this figure shows a type II process, characterized by a very thin axon with numerous small synaptic buttons. (C) Labeling with DSYT2. Note the presence of both type I (arrowhead) and type II (arrow) synapses with this muscle fiber.

at later stages of development. Fig. 8 shows Synaptotagmin staining in third instar larva. Synaptotagmin can be readily detected at synapses between nerve and muscle in the periphery. Neuromuscular junctions in the bodywall musculature of *Drosophila* have been classified into two categories based on button size and axonal branching (Johansen et al., 1989a). Type I projections are characterized by large buttons with few branches. Type II processes are much longer and thinner and have many smaller synaptic terminals along the muscle surface. Both type I (Fig. 8A,C) and type II (Fig. 8B,C) processes show Synaptotagmin immunoreactivity at synaptic terminals. Our antisera also labeled synapses in physically disrupted whole-mount third instar larval brains, revealing intense punctate labeling.

Ab. DSYT2 is also useful as a marker for synapses in the adult fly (see Fig. 9). The antibody shows reactivity throughout the neuropil in adult fly head sections with intense staining in the primary sensory centers like the antennal lobes (Fig. 9A,B,G) and visual lamina (Fig. 9C,D,E,H). Fig. 9G shows an enlarged view of the antennal lobes, where one can identify the glomerular-like islets (Strausfeld, 1976) where antennal nerve fibers synapse with dendrites of interneurons. Higher order synaptic stuctures such as the medulla, lobula and lobular plate, fan-shaped body and mushroom body also label. Control sections processed with preimmune sera or without addition of primary antibody lacked such staining. We therefore infer that Synaptotagmin maintains its synaptic localization throughout development and can be used as a synaptic marker at all stages of development.

DISCUSSION

Neurotransmitter release requires the action of numerous proteins at the presynaptic nerve terminal. A substantial number of these are associated with the synaptic vesicle and many have now been isolated and biochemically characterized (Sudhof and Jahn, 1991). Although specific roles for many of these proteins have been proposed on the basis of biochemical experiments, their function has not been established in vivo. One of these proteins, Synaptotagmin, has been shown to be highly conserved evolutionarily, exhibiting 57% sequence identity between rat and *Drosophila* homologues (Perin et al., 1991a). We have therefore chosen to examine Synaptotagmin expression during *Drosophila* development.

We find no evidence that *Drosophila* contains multiple Synaptotagmin isoforms encoded by different genes as observed in several vertebrate species (Geppert et al., 1991; Wendland et al., 1991). Our results suggest that there is only a single *syt* gene that encodes a single isoform of Synaptotagmin, simplifying the interpretation of the expression patterns discussed below and facilitating the future genetic dissection of *syt.* In situ hybridizations and immunocytochemical stainings show that *syt* is expressed in most, if not all, embryonic neurons, consistent with its hypothesized neuronal function. Hence, analysis of *syt* may provide insights into many aspects of neuronal function, synaptic vesicle trafficking and synaptic localization.

It has been shown that Synaptotagmin is localized

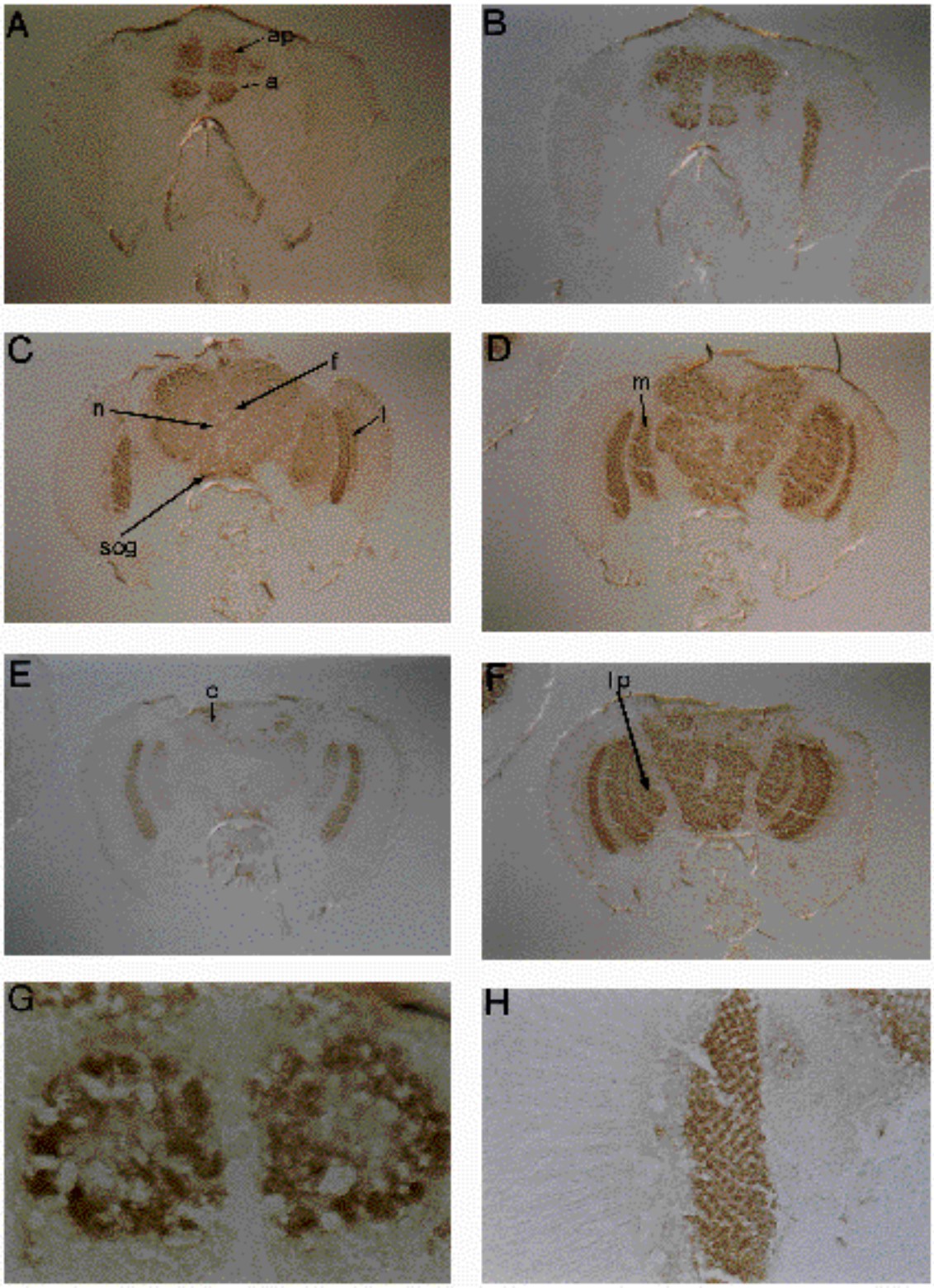


Fig. 9. Immunocytochemical staining of adult fly head sections stained with Ab. DSYT2 using a secondary antibody coupled to horse radish peroxidase. Sections have been arranged anterior to posterior (A-F) and dorsal up. Note the accumulation of synapses in the primary sensory centers of the antennal lobes (A,B,G) and visual lamina (C,D,E,H). The glomerular-like islets where the antennal nerve synapses onto interneuron dendrites is shown in G. The layered synaptic structure of the visual lamina is shown in H. Abbreviations: l, lamina; m, medulla; lp, lobula and lobular plate; c, mushroom body calyx; f, fan shaped body; n, noduli; sog, suboesophageal ganglion; a, antennal lobe; ap, anterior prominence of the inferior medial protocerebrum.

throughout rat hippocampal neurons in culture prior to synapse formation (Matteoli et al., 1992). Once synapses are formed between the neurons, rat Synaptotagmin becomes localized to synaptic contact sites. We first detect the presence of Synaptotagmin in *Drosophila* PNS neurites in stage 15 embryos. Muscle syncitia are known to form during stage 15, and the larval muscle pattern is completed by stage 16 (Johansen et al., 1989b; Broadie and Bates, 1993). The presence of Synaptotagmin in PNS axons at stages 15 and 16 suggests that synaptic vesicles are transported along axonal tracts before synapses are actually formed. By stage 16, when synapses begin to form between neurons and muscles, we see mobilization of Synaptotagmin to discrete compartments at the termini of axons. This time frame corresponds to the period during which Broadie and Bates (1993) were able to first detect post synaptic potentials in embryonic muscles. Thus, a similar distribution of synaptic vesicles occurs in vivo in *Drosophila* as in cultured hippocampal neurons, suggesting that the molecular mechanisms that specify synaptic vesicle localization to specific contact sites may be conserved throughout evolution. What role synaptic vesicles play prior to the formation of synapses is unclear. Matteoli et al. (1992) have shown that synaptic vesicles in the rat actually undergo cycles of exo-endocytosis in hippocampal neurons prior to localization at synapses, suggesting that synaptic vesicle exocytosis might play a role prior to synaptogenesis. In *Drosophila*, Synaptotagmin is also transiently present in the chordotonal dendrites in the PNS. By stage 17, this staining pattern is no longer detectable. It is thus likely that Synaptotagmin is transiently expressed in both dendritic and axonal compartments, with a subsequent signaling event that localizes synaptic vesicles to the synapse. Whether synaptic vesicles that are transiently present in dendrites, are redistributed to axons, or whether there is simply a barrier to further synaptic vesicle movement into dendrites is unknown.

Localization of Synaptotagmin protein provides a general marker allowing the localization of synaptic populations regardless of neurotransmitter content in the *Drosophila* embryo. This can most easily be illustrated in the PNS. The innervation pattern of bodywall muscle fibers by efferent motor axons, as revealed by Synaptotagmin expression, corresponds to that previously reported using immunocytochemistry with anti-glutamate and anti-HRP antibodies (Johansen et al., 1989b). However, in the CNS, little is known about the spatial distribution of the synapses. Interestingly, the intense staining along the longitudinal tracts of the VNC and the brain suggests that most synaptic connections are made in the vicinity of, or along these tracts. A portion of the staining along the longitudinal tracts could be due to transport of newly synthesized protein from the cell body to the synapse. However, the lack of detectable Synaptotagmin staining in the axons of stage 17 peripheral neurons projecting from and to the CNS suggest that transport of Synaptotagmin along axonal tracts is minimal and not easily detected by antibody staining after synapses are formed. In addition, the commissures of the CNS stain very faintly when compared to the longitudinal tracts. If transport were responsible for the strong staining along the longitudinal tracts, then commissures should stain equally strong. An additional possibility is that there is a preponderance of CNS neurons that are undergoing axonogenesis during stage 17 and thus the staining could represent transport along these tracts. This possibility is unlikely because we also detect intense staining along the VNC in larvae, when axonogenesis is completed. Indeed, single neuronal dye fills of embryonic motor neurons show dramatic arborizations of the axon along the longitudinal tracts, even after formation of its synaptic contact with muscle fibers (Sink and Whittington, 1991). We therefore conclude that most synapses are localized along the longitudinal tracts of the CNS. Alternatively, an additional Synaptotagmin protein exists which is specific for other CNS synapses and is not detected by our antibodies. Although we cannot rule out this possibility, we have been unable to find evidence for other *Drosophila* Synaptotagmins.

The compartmentalization of Synaptotagmin staining also demonstrates the timing of synapse formation in the PNS and, by analogy, the CNS. Synaptotagmin is first detected in stage 13 embryos in the CNS, just after axonogenesis is initiated (Campos-Ortega and Hartensten, 1985). During stages 14 and 15, Synaptotagmin accumulates outside the cell bodies in a punctate pattern along the tracts of the CNS, indicating connections among neurons of the CNS are formed during these stages. The targeting of synaptic vesicles to discrete sites occurs essentially simultaneously in all segments in the periphery during stage 16, suggesting that the molecular signals that specify synapse formation occur throughout the embryo in a synchronous fashion. The events that set up specific synapses at different muscles seem to be somewhat different for each neuromuscular junction in each segment since the shape and length of the synapses vary widely. However, specific synapses at similar muscles in different segments are very similar. The molecular mechanisms that are involved in axonal target selection and synapse formation are largely unknown. However, there is substantial evidence that a number of extracellular matrix proteins, secreted proteins and receptors may play crucial roles in synaptic targeting (Hunter et al., 1989; Nose et al., 1992; Reichardt and Tomaselli, 1991; Reist et al., 1992; Ushkaryov et al., 1992). It is possible that such proteins might also play a role in synaptic vesicle clustering, perhaps to cause local rearrangements of cytoskeletal elements at the contact site that might subsequently sequester synaptic vesicles to release sites. Alternatively, these proteins might participate in a signal transduction pathway that transports newly synthesized synaptic vesicles only to the synapse.

Synaptotagmin is also expressed in postembryonic stages as indicated by northern and western blots. This was confirmed by immunocytochemical staining of third instar larvae, which demonstrates that Synaptotagmin is present at synapses in both type I and type II processes of motor neurons. As expected, the CNS contains numerous synapses which can be most readily visualized in disrupted nervous systems. In adult fly head sections immunoreactivity is present throughout the neuropil of the CNS, with intense staining in the primary sensory terminations in the antennal lobe and visual lamina, as well as in higher order brain regions. Hence, the antisera that we have generated can be used at all stages of development to identify synaptic contact sites. These antisera are the first general synaptic markers to

be described in *Drosophila* and should therefore be an important tool to address questions concerning synaptic localization and function.

The temporal expression pattern and precise synaptic localization of Synaptotagmin in *Drosophila* and rat, along with the strong sequence similarity between the two proteins, suggest that Synaptotagmin plays a conserved role in synaptic function from invertebrates to vertebrates. In order to investigate the precise role of Synaptotagmin in neurotransmitter release, we have localized the *syt* gene to polytene chromosomal bands 23A6-23B1 and have begun a genetic analysis to obtain mutations in *syt*.

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REFERENCES

Bellen, H., Kooyer, S., D'Evelyn, D. and Pearlman, J. (1992). The *Drosophila* Couch potato protein is expressed in neural precursors and encodes nuclear proteins that contain RNA binding domains. *Genes Dev.* **6**, 2125-2136.

Bennett, M. K., Calakos, N. and Scheller, R. H. (1992). Syntaxin - A synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**, 255-259.

Bodmer, R., Carretto, R. and Jan, Y. N. (1989). Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages *Neuron* **3**, 21-32.

Broadie, K. E. and Bates, M. (1993). Development of the embryonic neuromuscular synapse of Drosophila melanogaster. *J. Neurosci.* **13**, 144-166.

Brose, N., Petrenko, A. G., Sudhof, T. C. and Jahn, R. (1992). Synaptotagmin - a calcium sensor on the synaptic vesicle surface. *Science* **256**, 1021-1025.

Budnick, V, Wu, C. -F., and White, K. (1989). Altered branching of serotonin-containing neurons in *Drosophila* mutants unable to synthesize serotonin and dopamine. *J. Neurosci.* **9**, 2866-2877.

Budnik, V., Zhong, Y., Wu, C. -F. (1990). Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J. Neurosci.* **10**, 3754-3768.

Campos-Ortega, J. A., and Hartenstein, V. (1985). *The Embryonic Development of* Drosophilamelanogaster. Berlin: Springer-Verlag.

Costello, W. J. and Wyman, R. J. (1986). Development of an indirect flight muscle in a muscle-specific mutnat of *Drosophila melanogaster*. *Dev. Biol.* **118**, 247-258.

Geppert, M., Archer III, B. T., and Sudhof, T. C. (1991). Synaptotagmin II: A novel differentially distributed form of synaptotagmin. *J. Biol. Chem.* **266**, 13548-13552.

Han, P. -L., Levin, L. R., Reed, R. R., and Davis, R. L. (1992). Preferential expression of the *Drosophila rutabaga* gene in mushroom bodies, neural centers for learning in insects. *Neuron* **9**, 619-627.

Hortsch, M., Bieber, A. J., Patel, N. H. and Goodman, C. S. (1990). Differential splicing generates a nervous system-specific form of *Drosophila* neuroglian. *Neuron* **4**, 697-709.

Hunter, D. D., Shah, V., Merlie, J. P., and Sanes, J. R. (1989). A laminin -like adhesive protein concentrated in the synaptic cleft of the neuromuscular junction. *Nature* **338**, 229-234.

Jan, L. Y. and Jan, Y. N. (1976). Properties of the larval neuromuscular junction in *Drosophila melanogaster*. *J. Physiol.* **262**, 189-214.

Johansen, J., Halpern, M. E., Johansen, K. M. and Keshishian, H. (1989a). Stereotypic morphology of glutamatergic synapses on identified muscle cells of *Drosophila* larvae. *J. Neurosci.* **9**, 710-25.

Johansen, J., Halpern, M. E. and Keshishian, H. (1989b). Axonal guidance and the development of muscle fiber-specific innervation in *Drosophila* embryos. *J. Neurosci.* **9**, 4318-32.

Koenig, J. H., Kosaka, T., and Ikeda, K. (1989). The relationship between the number of synaptic vesicles and the amount of transmitter released. *J. Neurosci.* **9**, 1937-1942.

Leveque, C., Hoshino, T., David, P., Shoji-Kasai, Y., Leys, K., Omori, A., Lang, B., El Far, O., Sato, K., Martin-Moutot, N., Newsom-Davis, J., Takahashi, M. and Seagar, M. J. (1992). The synaptic vesicle protein synaptotagmin associates with calcium channels and is a putative Lambert-Eaton myasthenic syndrome antigen. *Proc. Natl. Acad. Sci. USA* **89**, 3625-3629.

Matteoli, M., Takei, K., Perin, M. S., Sudhof, T. C., and DeCamilli, P. (1992). Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. *J. Cell Biol.* **117**, 849-861.

Nose, A., Mahajan, B. B., and Goodman, C. S. (1992). Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in *Drosophila*. *Cell* **70**, 553-567

Perin, M. S., Fried, V. A., Slaughter, C. A. and Sudhof, T. C. (1988). The structure of cytochrome b561, a secretory vesicle-specific electron transport protein. *EMBO J.* **7**, 2697-2703.

Perin, M. S., Fried, V. A., Mignery, G. A., Südhof, T. C. and Jahn, R. (1990). Phospholipid binding by a synaptic vesicle protein homologous to the regulatory domain of protein kinase C. *Nature* **345**, 260-263.

Perin, M. S., Archer, B. T., Ozcelik, T., Francke, U., Jahn, R. and Sudhof, T. C. (1991a). Structural and functional conservation of synaptotagmin (p65) in *Drosophila* and humans. *J. Biol. Chem.* **266**, 615-622.

Perin, M. S., Brose, N., Jahn, R., and Sudhof, T. C. (1991b). Domain Structure of synaptotagmin (p65). *J. Biol. Chem.* **266**, 623-629.

Petrenko, A. G., Perin, M. S., Davletov, B. A., Ushkaryov, Y. A., Geppert, M. and Sudhof, T. C. (1991). Binding of synaptotagmin to the α-latrotoxin receptor implicates both in synaptic vesicle exocytosis. *Nature* **353**, 65-68.

Reichardt, L. F. and Tomaselli, K. J. (1991). Extracellular matrix molecules and their receptors: Functions in neural development. *Annu. Rev. Neurosci.* **14**, 531-570.

Reist, N. E., Werle, M. J., and McMahan, U. J. (1992). Agrin released by motor neurons induces the aggregation of acetylcholine receptors at neuromuscular junctions. *Neuron* **8**, 865-868.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**, 5463-5468.

Sink, H. and Whittington, P. M. (1991). Pathfinding in the central nervous system and periphery by identified embryonic *Drosophila* motor axons. *Development* **112**, 307-316.

Sudhof, T. C. and Jahn, F. (1991). Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* **6**, 665-677.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Ushkaryov, Y. A., Petrenko, A. G., Geppert, M., and Sudhof, T. C. (1992). Neurexins: Synaptic cell surface proteins related to the α-latrotoxin receptor and laminin. *Science* **257**, 50-56.

Wendland, B., Miller, K. G., Schilling, J. and Scheller, R. H. (1991). Differential expression of the p65 gene family. *Neuron* **6**, 993-1007.

Zinn, K., McAllister, L. and Goodman, C. S. (1988). Sequence analysis and neuronal expression of Fasciclin I in grasshopper and *Drosophila*. *Cell* **53**, 577-87.

Zipursky, S. L., Venkatesh, T. R. and Benzer, S. (1985). From monoclonal antibody to gene for a neuron-specific glycoprotein in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **82**, 1855-1859.

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