Immunolocalization of Synaptotagmin for the Study of Synapses in the Developing Antennal Lobe of Manduca sexta

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ABSTRACT

In the mature olfactory systems of most organisms that possess a sense of smell, synapses between olfactory receptor neurons and central neurons occur in specialized neuropil structures called glomeruli. The development of olfactory glomeruli has been studied particularly heavily in the antennal lobe of the moth Manduca sexta. In the current study, we address the development of synapses within the antennal lobe of M. sexta by reporting on the localization of synaptotagmin, a ubiquitous synaptic vesicle protein, throughout development. A cDNA clone coding for M. sexta synaptotagmin was characterized and found to encode a protein that shares 67% amino acid identity with Drosophila synaptotagmin and 56% amino acid identity with human synaptotagmin I. Conservation was especially high in the C2 domains near the C-terminus and very low near the N-terminus. A polyclonal antiserum (MSYT) was raised against the unique N-terminus of M. sexta synaptotagmin, and a monoclonal antibody (DSYT) was raised against the highly conserved C-terminus of D. melanogaster synaptotagmin. In Western blot analyses, both antibodies labeled a 60 kD protein, which very likely corresponds to synaptotagmin. On sections, both antibodies labeled known synaptic neuropils in M. sexta and yielded similar labeling patterns in the developing antennal lobe. In addition, DSYT detected synaptotagmin-like protein in three other insect species examined. Analysis of synaptotagmin labeling at the light microscopic level during development of the antennal lobe of M. sexta confirmed and extended previous electron microscopic studies. Additional synapses in the coarse neuropil and a refinement of synaptic densities in the glomeruli during the last one-third of metamorphic development were revealed. J. Comp. Neurol. 441:277–287, 2001. © 2001 Wiley-Liss, Inc.

Indexing terms: synaptotagmin; development; synapses; olfactory system; glomeruli

In the mature olfactory system, synapses between peripheral receptor neurons and central olfactory neurons occur in specialized spheres of neuropil called glomeruli. Confinement of synaptic contacts from particular sensory neurons to specific glomeruli is believed to play an important functional role in organizing incoming olfactory sensory information (Lancet et al., 1982; Ressler et al., 1994; Sullivan and Dryer, 1996; Mustaparta, 1996; Hildebrand and Shepherd, 1997). Much current study is aimed at understanding how this glomerular organization develops and what is its role in olfactory perception. Glomerular organization is found in the primary olfactory neuropils of most vertebrate and invertebrate animals with a well-differentiated sense of smell (Boeckh et al., 1990; Hildebrand, 1996; Hildebrand and Shepherd, 1997). Thus, a

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Received 17 January 2001; Revised 6 July 2001; Accepted 19 September 2001
range of model systems, including simpler, more easily accessible developing systems, such as those of insects, can be used to study the formation of this specialized synaptic arrangement.

The moth Manduca sexta is a model system that has been used to elucidate many critical aspects of olfactory glomerulus development (see Oland and Tolbert, 1996). A thorough knowledge of where and when synapses form during glomerulus development will aid our understanding of the cellular interactions that give rise to these key structures. The driving aim of the current study was to elucidate synapse location and density throughout antennal lobe development by looking at synapses at the level of light microscopy. Electron microscopic sampling of the developing antennal lobe has revealed a changing distribution of synapses in the lobe at various stages of development (Tolbert, 1989; Oland et al., 1990), but these studies were limited by their ability to sample only very small regions of the developing antennal lobe at any one time.

To achieve our goal, we chose synaptotagmin, a ubiquitous synaptic vesicle protein, as a synapse marker for the developing antennal lobe, because antibodies against it have been used reliably in other invertebrate systems as markers for synapses (Littleton et al., 1993; Nonet et al., 1993; Hu, 1993). The gene for synaptotagmin has been cloned from a variety of species, including humans and Drosophila melanogaster (Perin et al., 1991a). The overall sequence organization of synaptotagmin is well conserved. All synaptotagmins cloned to date have a small, poorly conserved amino-terminal portion of the conserved carboxy-terminus of DSYT, a monoclonal antibody, was produced against a large piece of the conserved carboxy-terminus of D. melanogaster synaptotagmin; the other, M. sexta syt, is closely related to syt identified in other neuropil structures such as the glomeruli of the antennal lobe during the development of synapse-rich neuropil during the development of antennal lobe, because antibodies against it suggest that this protein will function similarly as an essential component of the synaptic machinery. Two antibodies were used for the immunocytochemical staining of antennal lobe at the light microscopic level. The cloned M. sexta syt is closely related to syt identified in other species and includes a well-conserved regulatory domain that suggests this protein will function similarly as an essential component of the synaptic machinery. Two different antibodies were used for the immunocytochemistry. One, MSYT, a polyclonal antiserum, was produced against a small piece of the poorly conserved amino-terminal portion of M. sexta synaptotagmin. The other, DSYT, a monoclonal antibody, was produced against a large piece of the conserved carboxy-terminus of D. melanogaster synaptotagmin. Both antibodies identify the same protein band at about 60 kD in Western blots and yield a similar labeling pattern throughout the development of the antennal lobe, confirming and extending the previous electron microscopic studies. Parts of this paper have been published in abstract form (Schachtner et al., 1999).

MATERIALS AND METHODS

Animals

M. sexta (Lepidoptera: Sphingidae) were reared on an artificial diet under a long-day photoperiod (L:D 17:7) at 26°C in walk-in environmental chambers. Pupae were staged according to the criteria described by Tolbert et al. (1983) and Jindra et al. (1997). The criteria involve changes in structures that are either superficial or readily visible through the pupal cuticle under a dissecting microscope. Tolbert et al. divide development into 18 stages (S1–S18) each of which lasts for 1–3 days, whereas Jindra et al. divide M. sexta pupal development into 21 “days” of development (P0–P20). Here we use the nomenclature of Jindra et al. (1997). For stages beyond P13 (S11), we used the staging criteria of Schwartz and Truman (1983).

RT-PCR

Primers were designed against the highly conserved amino acid sequences EAKNLKKM (5′-AGTNGCGDATNG-3′) and PIAQWHTL (5′-GARGCIALAARAYNTNAARATG-3′; Perin et al., 1991a; Nonet et al., 1993; Hu, 1993). The reverse and forward primers Synt1 and Synt2 were used to amplify M. sexta brain cDNA by polymerase chain reaction (PCR). Five micrograms of M. sexta whole-brain (stage 18) poly-A+ RNA were reverse transcribed following the protocol for the Superscript Choice cDNA Synthesis System (Gibco BRL, Grand Island, NY).

For PCR, 200 nM of all four dNTPs, 200 nM of both Synt1 and Synt2 primers, and 1 μl of cDNA were mixed with AmpliTaq reagents (Perkin-Elmer, Norwalk, CT), which included 2 mM magnesium chloride. After a 5 minute hot start at 94°C, 0.3 μl of AmpliTaq polymerase was added to the 20 μl reaction. Thirty cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds were performed on a 9600 thermocycler (Perkin-Elmer). The resulting ampiclon (~325 bp) was isolated by electrophoresis (Owl Scientific, Inc.) and TA cloned into the vector pCR 2.1 (Invitrogen, La Jolla, CA). Plasmids were sequenced, by an automated sequencing facility with an ABI sequencer. Sequence analysis was done with Geneworks v2.3 (Intelligenetics) for the Macintosh, and BLAST analysis was done using the NCBI website.

Library screening and sequencing

A single-strand, radioactively labeled probe was generated by PCR, using the linearized RT-PCR fragment as a template, 200 nM of an M13 reverse primer; 2 μM of magnesium chloride; 0.3 μl of AmpliTaq Gold (Perkin-Elmer); 200 mM dATP, dGTP, and dTTP; and 50 μCi of [32P]dCTP. Thirty-five cycles of 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 3 minutes were performed on a 9600 thermocycler (Perkin-Elmer). Unincorporated nucleotides were removed by ethanol precipitation. The probe was used to screen a M. sexta oligo-dT-prime P4 brain cDNA library under standard conditions (Sambrook et al., 1989). Two identical positive clones were obtained. The full-length sequence was obtained using exonuclease III deletion of both strands (Sambrook et al., 1989). Gaps in the exodetection sequence were filled by primer walking. Sequence analysis was done with Geneworks v2.3 for the Macintosh, and BLAST analysis was done using the NCBI website. Protein sequence alignments were done using Clustal W multiple sequence alignment (Thompson et al.,
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1994). This sequence was submitted to Genbank under accession number AF331039.

Northern blot analysis

Radiolabeled probes, generated as described above, were used to analyze Northern blots of 5 µg of poly-A+ RNA from P19 nerve cord, P4 brain, and P19 antenna. Total RNA from the various tissues was collected using Trizol (Gibco-BRL), and poly-A+ RNA was selected by a single pass over an oligo-dT column (Gibco-BRL). In addition, a probe against a 3’ untranslated region of M. sexta synaptotagmin, generated in the manner described above, was used to probe a Northern blot made from total RNA (20 µg/lane) from brain from six different stages of pupal development (P3, 4, 6, 8, 14, 20). Equal loading of the lanes was confirmed by quantification of the ethidium bromide staining of ribosomal RNA bands. Blots were hybridized in prehybridization solution [5× SSPE, 10% dextran sulfate, 50% formamide, 2% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, 10 mg salmon sperm DNA] at 42°C overnight. Blots were washed under standard conditions, with the final wash in 0.1× SSPE/1% SDS at 60°C.

Antibody production

**MSYT.** The polyclonal antisera, MSYT, was generated in rabbits against the synthetic peptide (RWRRE-AGPPETPVEESKEPE; residues 10–29 of DSYT. Purified recombinant D. melanogaster synaptotagmin (syt) protein containing residues 134–474, which corresponds to the conserved cytoplasmic sequence of synaptotagmin (Perin et al., 1991a), was used for making the syt monoclonal antibody (DSYT). The recombinant protein was produced in the pET system and dissolved in running gel buffer (pH 7.2) containing SDS. Three Balb/c mice were immunized with 10 µg of the recombinant protein, injected intraperitoneally in complete Ribi adjuvant. They were boosted three times at 2 week intervals. The sera from these injected mice were screened by immunocytochemical staining of grasshopper (Schistocerca americana) embryos at 50% development.

Western blot analysis

**SDS-PAGE.** Three M. sexta brains were split into six antennal lobes (AL), six optic lobes (OL), and three central brains, including the subesophageal ganglia (Br), and homogenized in 25 µl (AL) or 50 µl (OL, Br) phosphate-buffered saline (PBS), pH 7.4, containing phosphatase and protease inhibitors [0.5 mM Na3VO4, 0.5 mM phenylmethylsulfonyl fluoride (PMSF); both from Sigma, St. Louis, MO]. To 10 µl homogenate, 10 µl reducing sample buffer, pH 6.8, was added. After 3 minutes of boiling, samples were loaded onto a discontinuous SDS-polyacrylamide gel (3% stacking gel, 10% running gel) in a Bio-Rad (Richmond, CA) Mini-Protean 3 chamber and blotted to a nitrocellulose membrane (Hybond C; Amersham, Arlington Heights, IL), using a semidry transfer apparatus (Bio-Rad). To control the efficiency of protein transfer, nitrocellulose membranes were stained after transfer with Ponceau red.

**Immunoblot.** Blots were blocked in PBS (0.1 M) containing 0.05% Tween 20, 1% casein, and 20% fetal bovine serum for 1 hour and then incubated with the different antibodies at 4°C overnight (MSYT 1:10,000, DSYT 1:1,000) in PBS (0.1 M) containing 0.05% Tween 20 (PBST). For preabsorption, MSYT (1:10,000) was incubated for 30 minutes at room temperature with 34 µg/ml synthetic peptide. Blots were washed three times for 10 minutes in PBST, pH 7.4, 1% Triton X-100, and 1% SDS. Secondary antibodies [horseradish peroxidase (HRP)-coupled goat anti-mouse or goat anti-rabbit 1:5,000; Jackson Immunoresearch, West Grove, PA] were applied for 1 hour at room temperature in PBST. After washing, blots were developed by chemiluminescence using Supersignal substrate from Pierce (Rockford, IL) according to the instructions of the manufacturer. The signal was visualized with Fuji X-ray films, digitized, and further processed in Adobe Photoshop 5.0.2.

**Immunocytochemistry**

After dissection in cold saline (Weevers (1966) or PBS), brains of M. sexta, Schistocerca gregaria, or Leucophaea maderae were fixed in 4% PBS-buffered formaldehyde, pH 7.4, for 2 hours at room temperature or overnight at 4°C. After fixation, brains were embedded in gelatin/albumin and cut at 40 µm with a Vibratome (Leica VT 1000S) in the frontal plane. Prepupal D. melanogaster brains were processed for immunocytochemistry as described for M. sexta prepupae by Schachtner et al. (1998). D. melanogaster embryos were collected on agar-apple juice plates at 22°C, aged for the requisite times, and fixed with 4% formaldehyde. Antibody staining was carried out as described by Paululat et al. (1995). For the use of synaptotagmin antibody, instead of PBS, SST buffer (pH 7.4; see below) was used for the antibody incubation.

Vibratome sections were rinsed in 0.1 M Tris HCl (Sigma)/0.3 M NaCl (SST) with 0.1% Triton X-100 (SST-TX 0.1) for 1 hour at room temperature, then preincubated for another 1 hour with 5% normal donkey serum in SST-TX 0.5. Primary antibodies were then diluted in SST-TX 0.5 with 0.1% normal donkey serum. After incubation with the primary and secondary antibodies, sections were rinsed three times over 30 minutes in SST-TX 0.1 at room temperature and mounted on chromalum/gelatin-coated coverslips.

For all approaches, MSYT was used in a concentration of 1:10,000, DSYT in a concentration of 1:2,500. As secondary antisera, goat anti-rabbit or goat anti-mouse antibodies conjugated to HRP (1:500) or CY5 (1:500) were used for the antibody incubation. HRP was visualized with diaminobenzidine (DAB) using the glucose oxidase (Sigma) technique according to Watson and Burrows (1981). Tissues were then dehydrated in ethanol, cleared in xylene, and mounted in Entellan (Merck, Darmstadt, Germany). Sections were photographed with a Polaroid DMCe digital camera mounted on a Zeiss Axioskope. Fluorescence was detected using a confocal laser scanning microscope (Leica TCS-SP2). Images were then imported into Adobe Photoshop 5.0.2 and annotated in Microsoft PowerPoint.

**RESULTS**

**Cloning of M. sexta synaptotagmin (Msyt)**

Degenerate primer RT-PCR was used to identify a 325-base-pair fragment from M. sexta with high similarity to
previously identified synaptotagmins. This fragment was used to obtain a 5.5 kb *M. sexta* cDNA clone encoding synaptotagmin. This clone contains a long open reading frame of 1,287 base pairs and encodes a 429-amino-acid protein that is closely related to previously cloned synaptotagmins. We named this protein Msyt (for *M. sexta* synaptotagmin). Msyt shares 67% amino acid identity with *Drosophila* synaptotagmin and 56% amino acid identity with human synaptotagmin I, clearly placing it within the synaptotagmin family of molecules. This relationship is particularly pronounced in the conserved C2 domains (Perin et al., 1991a), where Myst shares 80% identity with *Drosophila* synt and 68% amino acid identity with human synaptotagmin I (Fig. 1). These domains are thought to regulate calcium-dependent interactions with the synaptic machinery. As in other species, the amino terminus is not well conserved and shows no significant sequence similarity (<20% amino acid identity) to any known sequence. This region is thought to be intravesicular. Thus, Msyt appears to be a well-conserved member of the synaptotagmin family with highly conserved calcium regulatory domains and a unique N-terminal intravesicular domain.

Kyte-Doolittle hydrophobicity analysis of Msyt reveals a single highly hydrophobic, potential membrane-spanning domain near the amino-terminus of the peptide in a location analogous to the transmembrane domains found in other synaptotagmins (Fig. 1). No recognizable signal sequence was found in the predicted peptide sequence, which also is true for *D. melanogaster* synaptotagmin (Perin et al., 1991a).

Examination of the expression patterns of Msyt mRNA using Northern blot analysis revealed the presence of an abundant 5.5 kb band in developing brain, nerve cord, and antenna of *M. sexta* (Fig. 2A). A closer examination of the developmental expression pattern in the developing brain demonstrated expression by pupal stage 3 and at all subsequent stages examined. The same abundant 5.5 kb band and two additional minor transcripts were detected in the brain. The minor transcripts appeared to be regulated in concert with the major band, insofar as all bands became more prominent with ongoing development (Fig. 2B). Thus, Msyt appears to be present throughout pupal development and might therefore be used as a marker for synapses in the developing nervous system.

**MSYT and DSYT recognize *M. sexta* synaptotagmin**

To localize Msyt protein during development, two antibodies were generated. MSYT, a polyclonal antibody, was generated against a synthetic peptide whose sequence was derived from the intravesicular amino-terminus of Msyt. This region is not well conserved among the other known synaptotagmins. For example, in the region used for antibody production (residues 10–29 of Myst), only one amino acid is identical between *M. sexta*, *D. melanogaster*, and human (Fig. 1).

The second antibody, DSYT, is a monoclonal antibody generated against the conserved carboxy-terminus of cloned *D. melanogaster* synaptotagmin (residues 134–474; Fig. 1). There is roughly 80% amino acid identity between Msyt and the *D. melanogaster* synt in this domain.

Immunoblotting of the same blot with DSYT and, after stripping, with MSYT showed that both antibodies recognize the same protein band at approximately 60 kD in *M. sexta* antennal lobe, optic lobes, and central brain including the subesophageal ganglion (Fig. 3A). Additionally, both antibodies labeled proteins of lower weight, for DSYT at about 33 kD and for MSYT at about 47 and 27 kD (Fig. 3A). Without stripping, MSYT recognizes more proteins in Western blot analysis (Fig. 3B). Preabsorption of MSYT with the synthetic 20 residue peptide of Msyt abolishes labeled protein bands at about 60, 41, and 27 kD, supporting the idea that the 60 kD band labeled by both antibodies contains Myst (Fig. 3C). However, after preabsorption, proteins with weights higher than 60 kD and a strong protein band at about 47 kD remained (Fig. 3C). In contrast, performing the same preabsorption experiment on 40 μm Vibratome sections resulted in no staining at all (Fig. 4A,B).

Comparison of MSYT and DSYT immunostaining on Vibratome sections revealed similar neuropil staining throughout the brain and antennal lobes of *M. sexta*, suggesting that both antisera label the same protein (cf. Figs. 4A and 5H). As expected, polyclonal MSYT produced more background staining than did monoclonal DSYT. The monoclonal antibody, generated against a well-conserved domain in synaptotagmin, also recognized synaptic neuropil in other species, including *L. madera*, *S. gregaria*, and *D. melanogaster* (Fig. 4).

Because DSYT was raised against a conserved domain of synaptotagmin and Myst against a unique amino acid sequence, DSYT should recognize synaptotagmin in all animal species, whereas MSYT should be specific for *M. sexta* synaptotagmin. We tested the antibody specificities in various insect species (Fig. 4). In adult *L. madera* and *S. gregaria* central nervous systems, DSYT labeled neuropil, whereas MSYT labeled nothing. In *D. melanogaster* embryos (E14) and white puparia (wp), DSYT labeled parts of the developing nervous system in a pattern similar to results previously published for a polyclonal antibody against the same region of *D. melanogaster* synaptotagmin (Littleton et al., 1993), confirming the specificity of this new monoclonal antibody (Fig. 4G,H). Surprisingly, MSYT produced light labeling in *D. melanogaster* embryos and wp, in areas not overlapping with labeling by DSYT (not shown). In *M. sexta*, however, MSYT and DSYT produced identical staining patterns (see below).

**Synapses in the developing antennal lobe**

We used both antisera to investigate the developmental time course of synaptotagmin staining in *M. sexta* antennal lobes. MSYT yielded the same labeling pattern during development as DSYT. Results using DSYT are shown in the figures because this antibody produced lower background staining. One day after pupal ecysis, P1, no synaptotagmin labeling was visible in the area of the developing AL (Fig. 5A). Early in metamorphosis, between P2 and P3, when all AL neurons had been born and shortly before the first sensory axons from the antenna reached the AL, a spheroidal neuropil area was labeled (Fig. 5B). Starting about 5 days after pupal ecysis (P5), syt labeling in the growing AL became concentrated in an outer shell area of the neuropil (Fig. 5C). Beginning on days P7/8, the stained shell area started to become organized into distinct stained glomeruli (Fig. 5D,E).

Glomeruli became increasingly distinct through P9 (Fig. 5F), and their labeling intensity peaked at P11/12 (Fig. 5H). By P16, the glomerular labeling had changed to a more differentiated labeling, suggesting areas of different synaptic density within each glomerulus (Fig. 5I).
At P7/8, faint, diffuse syt labeling appeared in olfactory receptor axons shortly before they enter the forming glomeruli (Fig. 5D,E). This diffuse labeling pattern became stronger up to P11/12 (Figs. 5F–H, 6A,B) and disappeared by P16 (Fig. 5I), when syt staining was localized mainly in the glomeruli. Interestingly, strong syt labeling was also visible as puncta in the central coarse neuropil of the AL, suggesting the presence of scattered synapses (Figs. 5H,I).

Fig. 1. Alignment of synaptotagmin amino acid sequences. Clustal W (Thompson et al., 1994) alignment of amino acid sequences from M. sexta, D. melanogaster, and human synaptotagmin I (Perin et al., 1991a). Identical residues are shaded. The C-terminal amino acids used for the RT-PCR are underlined. The peptide generated from the amino-terminal portion of M. sexta synaptotagmin (Msyt) for antibody production is marked with a dashed line. Msyt shares little identity with Drosophila synaptotagmin (Dsyt) or human synaptotagmin (Hsyt) in the amino-terminal (intravesicular) region. The transmembrane regions of Hsyt and Dsyt and the corresponding hydrophobic region of Msyt are boxed (Perin et al., 1991a). There is little sequence identity between Msyt, Dsyt, and Hsyt transmembrane regions in contrast to the strong sequence identity found in this region among mammalian synaptotagmins (Perin et al., 1991a). The carboxy-terminal domains, C2A and C2B, which are similar to regulatory domains of protein kinase C, are indicated with a line above them (Perin et al., 1991a). C2 domains are highly conserved among different synaptotagmins, including Msyt (Perin et al., 1991a; Nonet, 1993). Msyt C2 domains are 89% and 76% identical to Dsyt and Hsyt C2 domains, respectively.

At P7/8, faint, diffuse syt labeling appeared in olfactory receptor axons shortly before they enter the forming glomeruli (Fig. 5D,E). This diffuse labeling pattern became stronger up to P11/12 (Figs. 5F–H, 6A,B) and disappeared by P16 (Fig. 5I), when syt staining was localized mainly in the glomeruli. Interestingly, strong syt labeling was also visible as puncta in the central coarse neuropil of the AL, suggesting the presence of scattered synapses (Figs. 5H,I).
This staining of the coarse neuropil became visible initially at P8 and remained into adulthood.

**DISCUSSION**

*M. sexta* synaptotagmin

We isolated a 5.5 kb synaptotagmin cDNA clone from *M. sexta*. This clone appears to be full-length, in that it closely matches the size of the major transcript seen on Northern blots and contains a complete open reading frame with an initiator methionine and multiple stop codons both up- and downstream. The open reading frame encodes a protein of 429 amino acids with high sequence similarity to synaptotagmins previously identified from other species. The sequence similarity is especially pronounced in the carboxy-terminal C2 domains that are thought to mediate the calcium regulation of synaptotagmin interactions with the synaptic machinery. The combined evidence suggests that Msyt is a new member of the synaptotagmin family and is likely to mediate synaptic interactions in the *M. sexta* nervous system.

Northern blot analysis revealed that a 5.5 kb transcript is present in developing brain, nerve cord, and antenna of *M. sexta*. Two smaller transcripts also were seen in brain, suggesting the possibility of multiple splice variants. Splice variants are not commonly reported for mammalian synaptotagmins, but multiple transcripts differing in the 3’ untranslated region were identified for *D. melanogaster* synaptotagmin (Perin et al., 1991a).

**Specificity of the antibodies for synaptotagmin**

To maximize confidence in our analyses of synapses in the developing AL, a polyclonal antibody generated against a unique sequence in *M. sexta* synaptotagmin and a monoclonal antibody generated against a well-conserved synaptotagmin domain in *D. melanogaster* were used for both Western blots and immunocytochemistry. Both antibodies recognized the same 60 kD band on immunoblots, and each separately also recognized other bands. The 60 kD band recognized by MSYT was eliminated by preadsorption with synthetic Msyt peptide. Analysis of the *M. sexta* synaptotagmin clone predicts a protein size of only 42.9 kD. On Western blots, syt from rat and *D. melanogaster* have been shown to be larger than their predicted sizes; at least some of this greater size is attributed to N-linked glycosylation in the rat (but not in *D. melanogaster*; Perin et al., 1991b; Littleton et al., 1993). The predicted amino acid sequence for Msyt has potential sites for N-linked glycosylation, raising the possibility...
that this and perhaps other types of posttranslational modification could be increasing the actual size of the protein.

In immunocytochemistry, DSYT labeled the same synaptic neuropils in *D. melanogaster* that were recognized by a previously characterized *D. melanogaster* polyclonal antiserum against syt (Littleton et al., 1993), suggesting that both recognize the same molecules. In adult *M. sexta*, both MSYT and DSYT clearly labeled well-described synaptic neuropils, such as the OL neuropils, the central complex, and the glomeruli of the AL. They also both produced the same labeling patterns in *M. sexta* AL during development, and this common labeling could be eliminated by preadsorption with syt. Further evidence that MSYT labels syt comes from studies of the neuromuscular junction in *M. sexta*. There, immunoreactivity has been shown to colocalize precisely with regions of synaptic vesicle recycling that were labeled by extracellular application of the lipophilic dye FM-143 (Consoulas and Levine, 1998). In addition to strong punctate labeling of the FM-143 uptake sites, light, diffuse labeling was seen in the shafts of regenerating axons, similar to that we saw in olfactory receptor axons at certain developmental stages (Figs. 5D–H, 6A,B), presumably as a result of synaptotagmin being transported down the axons (see below). Recently, MSYT was also shown to produce strong punctate labeling of synapses in the circulatory system of *M. sexta* (Davis et al., 2001). These results strongly suggest that our syt antibodies strongly label synapses in the peripheral and central nervous systems of *M. sexta*. The common labeling pattern obtained with both MSYT and DSYT and the ability to block that labeling by preabsorption of the antibody with syt and the similarity of DSYT staining in *D. melanogaster* to that obtained using a previously characterized antiserum give us confidence that the immunocytochemical labeling reported here represents genuine and specific *M. sexta* synaptotagmin.

**Localization of synaptotagmin to synapses**

Synaptotagmin is closely associated with synaptic vesicles in both vertebrate and invertebrate systems (Perin et al., 1991b; Nonet et al., 1993; Hu, 1993). In adult rat, syt was completely removed from brain homogenates by immunoprecipitation of synaptic vesicles with an antibody to synaptophysin, another synaptic vesicle protein (Perin et al., 1991b). In both *D. melanogaster* and *C. elegans*, syt was shown to be restricted to synaptic regions of the nervous system (Littleton et al., 1993; Nonet et al., 1993).

In addition to their localization on synaptic vesicles, vertebrate synaptotagmins I/II are expressed in neuronal growth cones (Igarashi et al., 1997; Kabayama et al., 1999), which differentiate into presynaptic terminals after reaching their targets (Hall and Sanes, 1993). In the growth cone, syt seems to be involved in the process of growth, in that blocking the C2A domain, which, together with the C2B domain, is responsible for the Ca$^{2+}$ sensitivity of synaptotagmin (Chapman and Davis, 1998; Desai et al., 2000), significantly inhibits neurite outgrowth in cultured chick dorsal root ganglion neurons (Kabayama et al., 1999). Recent experiments also show that expression of synaptotagmin I/II promotes neurite outgrowth in PC 12 cells (Fukuda and Mikoshiba, 2000).

Perhaps related to growth cone localization, syt is transiently localized in neuronal cell bodies in the developing *D. melanogaster* nervous system. It is then very rapidly transported to forming synapses during genesis of the neurites (Littleton et al., 1993). In the current study, we have shown both strong punctate labeling, presumably at synapses, and faint, diffuse labeling in growing axons (Fig. 6). We interpret this diffuse labeling as nonsynaptic localization of syt in growth cones or in axon shafts as syt is transported to axon terminals.
The ability of our syt antibodies to label synapses allowed us to examine the development of the synapse-rich glomerular neuropil of the AL at the level of light microscopy for the first time. The results correlate well with previous electron microscopic observations (Tolbert, 1989; Oland et al., 1990) but extend them and add new details concerning synapse distribution.

As summarized in Figure 7, before olfactory receptor axons innervated the lobe (before P3), dense but punctate syt labeling was scattered throughout the neuropil (Fig. 7A), almost certainly reflecting the presence of synapses seen by Tolbert (1989) to occur between the neurites of AL neurons. As receptor axons entered the lobe (from P3) and the lobe grew in size, syt density increased in the outer rim of the neuropil, where fine branches of AL neurons were growing and forming more synapses (Tolbert, 1989; Oland et al., 1990). Simultaneously, syt labeling decreased in the center of the neuropil, which, by then, was dominated by thicker branches of the neurites of AL neurons (Oland et al., 1990; Fig. 7B). As receptor axons began to form protoglomeruli (P5/6; Oland et al., 1990), dense syt labeling became concentrated in the protoglomeruli as discussed above (Fig. 7C).

Visualization of synapses in the developing AL

The results of our visualization allowed us to examine the development of the synapse-rich glomerular neuropil of the AL at the level of light microscopy for the first time. The results correlate well with previous electron microscopic observations (Tolbert, 1989; Oland et al., 1990) but extend them and add new details concerning synapse distribution.

As summarized in Figure 7, before olfactory receptor axons innervated the lobe (before P3), dense but punctate syt labeling was scattered throughout the neuropil (Fig. 7A), almost certainly reflecting the presence of synapses seen by Tolbert (1989) to occur between the neurites of AL neurons. As receptor axons entered the lobe (from P3) and the lobe grew in size, syt density increased in the outer rim of the neuropil, where fine branches of AL neurons were growing and forming more synapses (Tolbert, 1989; Oland et al., 1990). Simultaneously, syt labeling decreased in the center of the neuropil, which, by then, was dominated by thicker branches of the neurites of AL neurons (Oland et al., 1990; Fig. 7B). As receptor axons began to form protoglomeruli (P5/6; Oland et al., 1990), dense syt labeling became concentrated in the protoglomeruli as discussed above (Fig. 7C).

As previously shown with axon staining methods, the formation of glomeruli proceeds in a wave from the dorsolateral to the ventromedial part of the AL, with the sex-specific glomeruli developing first (Malun et al., 1994; Rössler et al., 1998). The synaptotagmin labeling mirrored this pattern exactly, underscoring the usefulness of this marker for studying overall glomerulus development. In Figure 5D,E, the two large glomeruli at the entrance of the antennal nerve, the lateral female glomeruli (LFGs), were...
the first to develop and the first to be labeled by our
antibodies. As development proceeds, glial cells encircle
the forming glomeruli (Oland and Tolbert, 1987; Oland et
al., 1990; Malun et al., 1994; Ro¨ssler et al., 1998) and the
intensity of synaptic labeling increases in a wave from the
inner to the outer edge of individual glomeruli (Fig. 7D).
These changes in labeling intensity suggest an increase in
formation of synapses between olfactory receptor axons
and AL neurons within the confines of the glomeruli and
had not been appreciated in the previous electron micro-
scopic studies.

Additionally, punctate syt staining occurred in the
course neuropil deep to the developing glomeruli at about
P8, indicating the presence of synapses in this area. Pre-
vious studies reported small numbers of ultrastructurally
identified synapses in the coarse neuropil (Tolbert and
Hildebrand, 1981; Tolbert, 1989), with the preponderance
of synapses occurring within glomerular borders. The cur-
rent light microscopic study, providing a more complete, if
lower resolution, view of synapse distribution, showed
that the number of synapses in the coarse neuropil of the
mature lobe, though still small compared with the number
in glomeruli, is substantial.

The syt antibodies that we have generated are useful
tools for the study of synaptic neuropils in
M. sexta.

![Fig. 6. Frontal Vibratome section through the antennal lobe of a
P10 male. Each row (A–C) shows in column 1 a Normarski contrast
image, in column 3 a DSYT antibody label (red; CY5), and in column
2 an overlay of 1 and 3. A: Overview, dashed boxes in A3 relate to
the areas in rows B and C. Arrowheads in A1 mark the area of normal
glomeruli (black) and the male-specific macroglomerular complex
(MGC; white). B: Higher magnification of MGC area. The strong,
punctate syt labeling is restricted to the glomeruli (cf. B1–3), whereas
the faint diffuse staining refers to the preterminal axon shafts of the
incoming olfactory receptor neurons (ORNs). Dashed line in B1
marks the border between glomerular (GL) and ORN area. C: Coarse
neuropil (CN) in the center of the antennal lobe. Arrows show strong
punctate labeled areas within the CN. LC, lateral cell group; MC,
medial cell group; Scale bars = 80 μm in A, 40 μm in B, 20 μm in C.

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The syt antibodies that we have generated are useful
tools for the study of synaptic neuropils in M. sexta. For
the olfactory system, these antibodies will significantly
enhance our ability to observe gross changes in synaptic
patterns in AL that are perturbed experimentally during
development. In addition, the antibodies may be useful in
dissociated-cell and organ-culture preparations. Overall,
our increased ability to localize synaptic sites at the light
microscopic level will allow a better understanding of the
role and regulation of synapses in the developing and mature olfactory system.

ACKNOWLEDGMENTS

The authors thank Dr. Achim Paululat for providing D. melanogaster embryos and prepupa and for his support in immunostaining D. melanogaster embryos. The authors also thank Dr. Nicholas Gibson for his insight and technical assistance and Dr. A.A. Osman for assistance in animal rearing. The study was supported by grants from the National Institutes of Health to L.P.T. (NS20040) and animal rearing. The study was supported by grants from the Deutsche National Institutes of Health to L.P.T. (NS20040) and animal rearing. The study was supported by grants from

LITERATURE CITED


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