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Localization of Choline Acetyltransferase-Expressing Neurons in *Drosophila* Nervous System

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ABSTRACT A variety of approaches have been developed to localize neurons and neural elements in nervous system tissues that make and use acetylcholine (ACh) as a neurotransmitter. Choline acetyltransferase (ChAT) is the enzyme catalyzing the biosynthesis of ACh and is considered to be an excellent phenotypic marker for cholinergic neurons. We have surveyed the distribution of choline acetyltransferase (ChAT)-expressing neurons in the *Drosophila* nervous system detected by three different but complementary techniques. Immunocytochemistry, using anti-ChAT monoclonal antibodies results in identification of neuronal processes and a few types of cell somata that contain ChAT protein. In situ hybridization using cRNA probes to ChAT messenger RNA results in identification of cell bodies transcribing the ChAT gene. X-gal staining and/or β-galactosidase immunocytochemistry of transformed animals carrying a fusion gene composed of the regulatory DNA from the ChAT gene controlling expression of a *lacZ* reporter has also been useful in identifying cholinergic neurons and neural elements. The combination of these three techniques has revealed that cholinergic neurons are widespread in both the peripheral and central nervous system of this model genetic organism at all but the earliest developmental stages. Expression of ChAT is detected in a variety of peripheral sensory neurons, and in the brain neurons associated with the visual and olfactory system, as well as in neurons with unknown functions in the cortices of brain and ganglia. *Microsc. Res. Tech. 45:65–79, 1999.*

INTRODUCTION

Acetylcholine (ACh) is an important major neurotransmitter in the nervous system of most animals, including insects. ACh, along with its biosynthetic enzyme choline acetyltransferase (ChAT, EC 2.3.1.6) and degradative enzyme acetylcholinesterase (AChE, EC 3.1.1.7), is present in *Drosophila* at very high levels when compared to brain regions from a number of vertebrates (for reviews see: Buchner, 1991; Restifo and White, 1990; Salvaterra and Vaughn, 1989). Null mutations in either the ChAT or AChE genes result in late embryonic lethality (Greenspan, 1980; Hall and Kankel, 1976) indicating the essential nature of both genetic functions. The genes for *Drosophila* ChAT (*Cha*) and AChE (*Ace*) have both been cloned and characterized (Hall and Spierer, 1986; Itoh et al., 1986; Sugihara et al., 1990). Regulatory DNA has been mapped in *Drosophila* adults and at earlier developmental stages for *Cha* by constructing transgenic animals where the 5' flanking DNA is used to drive reporter gene expression (Kitamoto et al., 1992, 1995; Kitamoto and Salvaterra, 1993). Conditional temperature-sensitive mutants of either *Cha* or *Ace* show morphological, physiological, and behavioral abnormalities in animals challenged at a restrictive temperature (Greenspan, 1980; Gorczyca and Hall, 1984; Chase and Kankel, 1988). These genetic observations imply that cholinergic transmission is important for a variety of neurobiological processes in this animal. The genes encoding subunits of the two main classes of ACh receptors, nicotinic and muscarinic, have also been identified and characterized in *Drosophila* (for reviews see: Gundelfinger, 1992; Gundelfinger and Hess, 1992; Hannan and Hall, 1993; Trimmer, 1995). Recently, the gene encoding *Drosophila* vesicular acetylcholine transporter protein has also been identified and shown to be organized along with ChAT into a cholinergic locus similar to the genomic organization seen in other species (Kitamoto et al., 1998). This wealth of genetic and molecular information on genes related to ACh metabolism has established *Drosophila* as an excellent model system to study the function and regulation of cholinergic neurotransmission in a comprehensive manner.

In spite of the wealth of genetic and molecular information about *Drosophila* cholinergic macromolecules and genes, physiological studies characterizing the function of cholinergic transmission are still rare (Gorczyca and Hall, 1984; Gorczyca et al., 1991; Greenspan, 1980). One reason for this is that few specific cholinergic neurons have been directly identified. For further analysis and characterization of the function of cholinergic transmission, it is necessary to obtain a more detailed map of cholinergic systems in

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Drosophila and identify particular neurons that may be favorable for future physiological analysis. This review briefly summarizes our present knowledge of the distribution of cholinergic cells in *Drosophila* nervous system.

ChAT has long been considered the most reliable biochemical marker for cholinergic neurons (Salvaterra and Vaughn, 1989). Three monoclonal antibodies specifically recognizing *Drosophila* ChAT protein (1C8 and 1G4: Crawford et al., 1982; 4B1: Takagawa and Salvaterra, 1996) have been used for immunocytochemical studies. The results have revealed a wide distribution of ChAT containing neural elements in specific regions of the neuropil in the adult and larval nervous system (Buchner et al., 1986; Gorczyca and Hall, 1987; Ikeda and Salvaterra, 1989; Yasuyama et al., 1995a,b, 1996a). It has not been possible using immunocytochemistry, however, to definitively localize the cell bodies contributing to most ChAT immunoreactive terminals. In only a few specialized cases, have cell bodies been identified (Gorczyca and Hall, 1987; Yasuyama et al., 1995a,b, 1996a).

An alternative approach to identify neurons expressing ChAT is in situ hybridization using cRNA probes specific for *Drosophila* ChAT mRNA. ChAT gene transcripts have been localized in nearly the entire cortical region of the cephalic ganglion using this technique (Barber et al., 1989; Yasuyama et al., 1996a). Unfortunately, this approach is insufficient to provide single-cell resolution of most cells primarily because of the small size of *Drosophila* cortical interneurons. A subset of labeled larger cell bodies, however, was detectable in the cortices associated with the optic and antennal lobes.

A third reporter gene approach has also been used to locate *Drosophila* cholinergic neurons. ChAT regulatory DNA is fused in frame with a β-galactosidase reporter gene and used to generate transgenic fly lines with P-element technology. Histochemical and/or immunocytochemical detection of β-galactosidase reporter gene, whose expression is controlled by ChAT regulatory DNA, can thus reveal cholinergic neurons. A series of P-element mediated transformed fly lines containing approximately 7.4 kb of 5' flanking DNA from the *Drosophila Cha* gene (7.4 kb-ChAT/*lacZ* transformants) have been generated by Kitamoto et al. (1992). These transformants display a β-galactosidase expression pattern that is very similar to that of endogenous ChAT protein in adult animals (Kitamoto et al., 1992, 1995) as well as earlier developmental stages (Kitamoto and Salvaterra, 1993). One advantage of this approach to localizing cholinergic neurons is that the distribution of X-gal reaction product is not confined to the neuropil, as seen for ChAT immunocytochemistry, or to the cell bodies, as seen with in situ hybridization, but can be observed throughout the neurons.

The aim of the present review is to summarize previous observations on the distribution of cholinergic neurons in *Drosophila* central and peripheral nervous system inferred from these three distinct but complimentary localization techniques. Comprehensive information on cholinergic as well as other neurotransmitter systems in *Drosophila* and other insects can be obtained from the excellent reviews by Buchner (1991), Nässel (1991) and, Restifo and White (1990).

DISTRIBUTION OF PUTATIVE CHOLINERGIC NEURONS IN PERIPHERAL NERVOUS SYSTEM

ChAT expression is exclusively inferred in chemosensory neurons associated with the olfactory and gustatory system, and proprioceptive sensory neurons by X-gal staining of P-element transformants carrying a 7.4 kb/*lacZ* fusion gene. In adults, photoreceptor cells in the ocelli and the compound eyes, as well as the mechanosensory neurons associated with tactile bristles, are not stained. The lack of staining in these cells is consistent with the suggestion that histamine, not ACh, is the synaptic transmitter used by photoreceptors and mechanosensory cells in *Drosophila* (Buchner et al., 1993; Pollack and Hofbauer, 1991). In contrast to adults, the larval photoreceptor organ (Bolwig's organ; Bolwig, 1946) expresses the ChAT gene, suggesting that ACh may function as a neurotransmitter in the larval photoreceptor cells (Yasuyama et al., 1995b).

Embryos

In stage-15 embryos, the peripheral sensory neurons are distributed in four loose clusters within each abdominal segment, from ventral to dorsal termed: *v*, *v'*, *l*, and *d* (Ghysen et al., 1986). β-Galactosidase expression is apparently found in all sensory neurons of the *d* cluster, whereas in the *l* and *v* clusters, some of the neurons such as the chordotonal neurons do not show positive reporting (Kitamoto and Salvaterra, 1993). In situ hybridization using a nonradioactive ChAT cRNA probe, strongly labels three pairs of cell clusters: Bolwig's organs, the dorsal and ventral organs in the gnathocephalon of the embryo, and clustered sensory neurons in abdominal segments (Yasuyama et al., 1995b).

Larvae

The larval photoreceptor organs (Bolwig' organs), which lie lateral to the mouth hooks, project their axons to the brain via Bolwig's nerves through the optic stalks (Bolwig, 1946; Steller et al., 1987). In the third larval instar, Bolwig's organs are strongly stained with X-gal. Bolwig's nerve running through the optic stalk also exhibits strong ChAT immunoreactivity (Yasuyama et al., 1995b). In the imaginal leg discs of late third instar larvae, β-galactosidase expression can be observed. The six to seven cell bodies near the center of the leg discs are stained (Kitamoto and Salvaterra, 1993) with a similar pattern of distribution as the pre-existing neurons described by Jan et al. (1985), suggesting these stained cells are sensory neurons. No β-galactosidase expression is detectable in the eye, antenna, or wing discs. The distribution of sensory neurons expressing the ChAT gene in larval peripheral nervous system remains to be examined.

Adults

Antenna. The third segment (funiculus) bears the olfactory sensilla classified into three morphological types: club-shaped basiconic sensilla, spine-shaped trichoid sensilla, and small cone-shaped coeloconic sensilla. These sensilla are arranged in a characteristic pattern on the surface (Mindek, 1968; Stocker et al., 1983; Stocker, 1994). Most, if not all, of the sensory neurons associated with these three types of sensilla

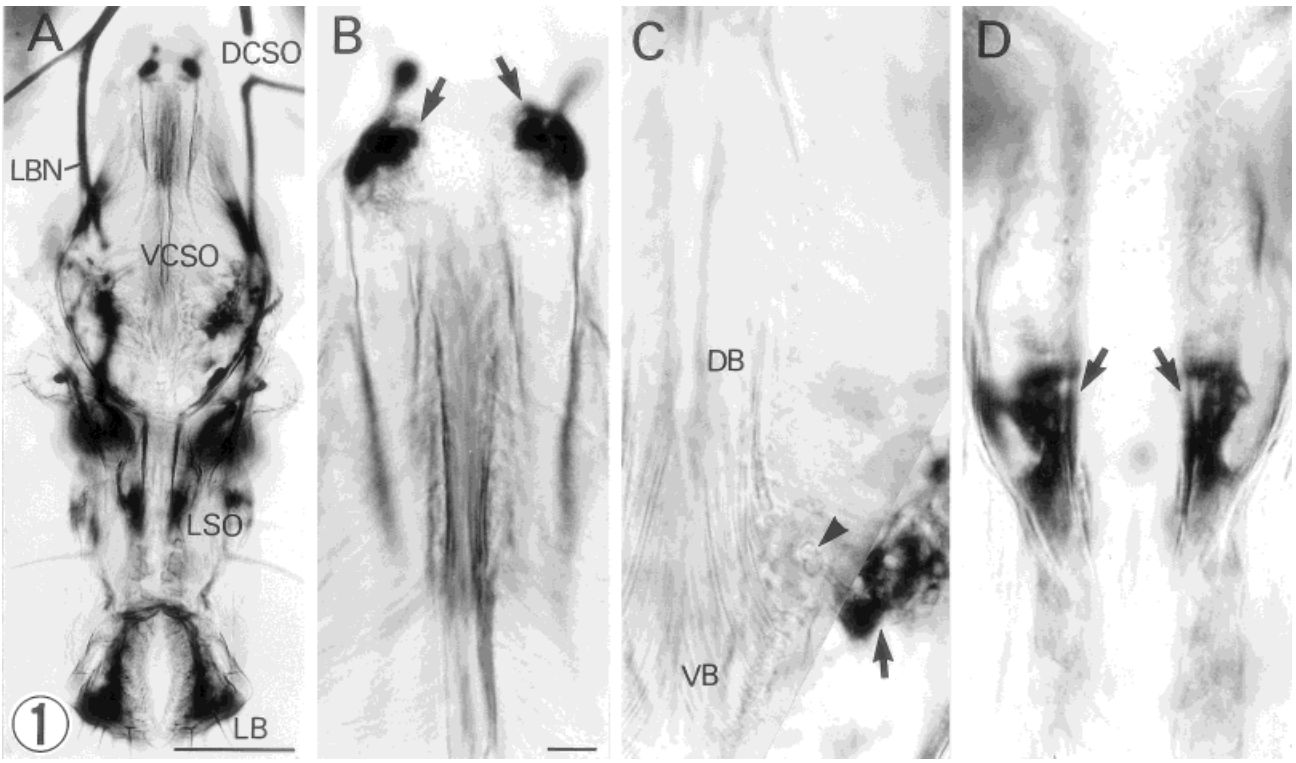

Fig. 1. Distribution of X-gal staining in the pharynx of adult 7.4 kb-ChAT/*lacZ* transformants. **A:** Three groups of paired cell body clusters associated with the sense organs (DCSO, VCSO and LSO) are stained along the pharynx. B–D: Magnified views of the cell body clusters (arrows). **B:** The dorsal cibarial sense organs (DCSO). **C:** The ventral cibarial sense organs (VCSO; arrowhead). **D:** The labral sense organs (LSO). DB, VB, dorsal and ventral fish-trap bristles; LB, labial palps; LBN, labial nerve. Scale bar in A = 100 µm; in B =10 µm.

are stained with X-gal (Kitamoto et al., 1995). In the second segment (pedicel), the sensory neurons of Johnston's organ, which is presumably a detector of vibratory stimuli (Ewing, 1978; Miller, 1950), are also stained. In the arista, a faintly stained bundle is detectable (Kitamoto et al., 1995).

Maxillary Palp. This appendage bears two categories of sensillum that can be distinguished: olfactory sensilla of the basiconic type and mechanosensory bristles (Singh and Nayak, 1985; Stocker, 1994). Staining with X-gal is mainly detected in the cell bodies located on the dorsal side of the palps, where the olfactory sensillum cover the surface, whereas the mechanosensory bristles are located on the ventral surface. This suggests that the sensory neurons associated with the olfactory sensilla are expressing reporter gene and are thus likely to be cholinergic (Kitamoto et al., 1995).

Labial Palps. The labellum contains two major types of sensillum: taste bristles and taste pegs (Stocker, 1994). Staining with X-gal is detected in the sensory cells of all these sensilla. In addition, a distinct cell body that may correspond to the multipolar cell described by Wilczek (1967) in the blowfly, is stained in each half of the labial palps close to the labial nerve (Kitamoto et al., 1995).

Pharynx. Three groups of paired cell body clusters are stained with X-gal along the pharynx (Fig.1A). These cell clusters seem to belong to the dorsal and ventral cibarial sense organs, and the labral sense organ, respectively. Each of the dorsal cibarial sense organs contains two sensilla with three chemosensory neurons (Nayak and Singh, 1983). Three stained cell bodies are detected in a cluster dorsally adjacent to this sense organ, and likely to be its sensory neurons (Fig.1B). Each ventral cibarial sense organ has two or three sensilla with two to four chemosensory neurons (Nayak and Singh, 1983). A cluster of stained cell bodies, which likely consists of three or more cell bodies, is found close to each ventral cibarial sense organ (Fig.1C). The cluster seems to be associated with this sense organ, but not a ventral and a dorsal row of "fish-trap" bristles (Nayak and Singh, 1983; Stocker and Schorderet, 1981), which are mononeuronal mechanosensory bristles (Nayak and Singh, 1983). The labral sense organ consists of nine sensilla forming an irregular row on either side of the midline of pharynx behind the oral opening (Nayak and Singh, 1983). Kitamoto et al. (1995) found at least four stained cells on each side of the midline in cryosections at the level of this organ (see also Fig.1D).

Legs. The sensilla in the legs are categorized into three types: tactile bristles, which are abundantly distributed over the entire leg surface; taste bristles, most of which are concentrated on the tarsal segments; and internal and external proprioceptors, including the hair plate, campaniform sensilla, and chordotonal organs (e.g., Kankel et. al., 1980; Murphey et al., 1989; Nayak and Singh, 1983; Shanbhag et al., 1992; Smith and Shepherd, 1996; Stocker, 1994). Figure 2 summa-

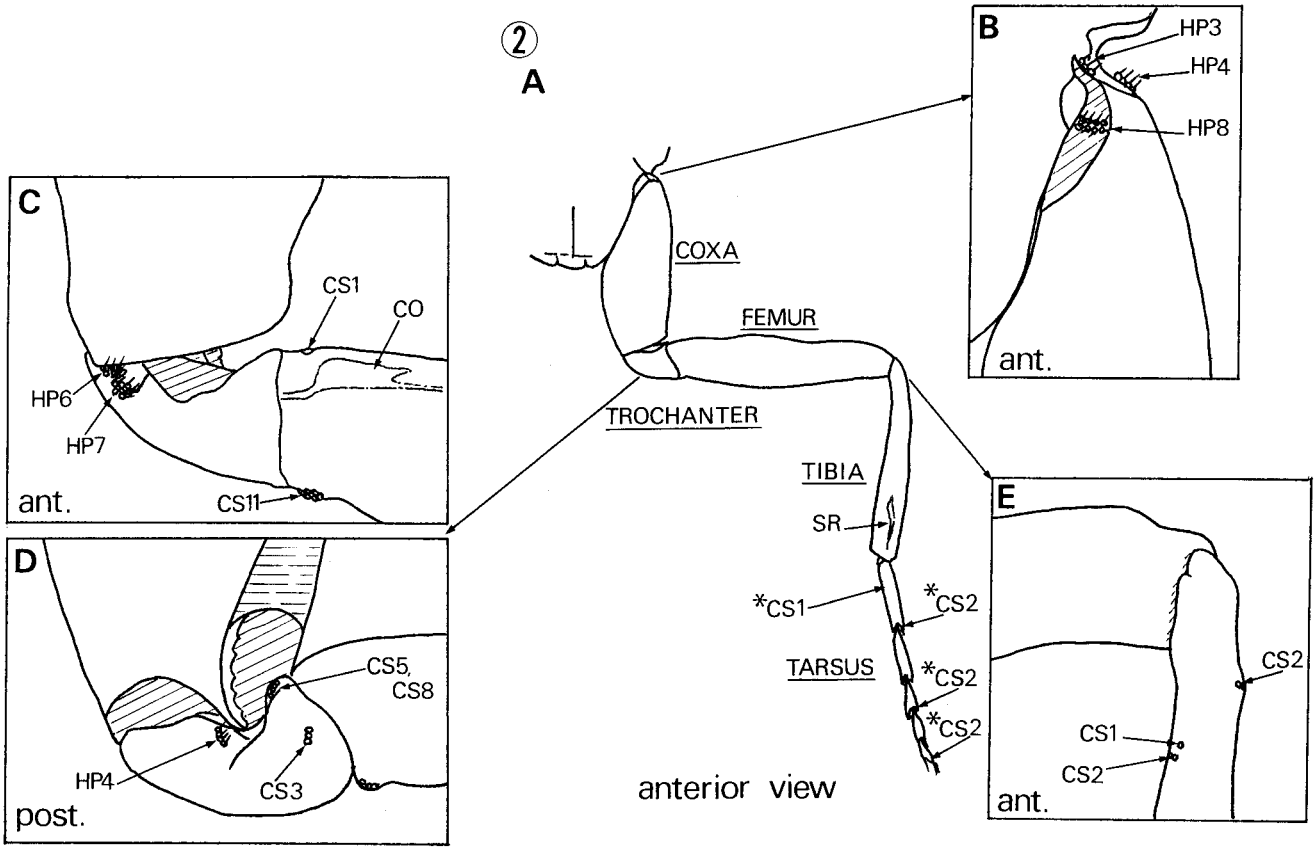

Fig. 2. Diagrams of the positions of proprioceptors on an adult prothoracic leg. **A–C,E:** Anterior view. **D:** Posterior view. CO, chordotonal organ; CS, campaniform sensillum; HP, hair plate; SR, stretch receptor. Each number appended to CS or HP represents the number of sennsillum composing each cluster of hair plates or campaniform sensilla.

rizes the distribution of proprioceptors on a prothoracic leg stained with X-gal. Staining is restricted to the sensory neurons associated with the taste bristles and proprioceptors (Kitamoto et al., 1995; Yasuyama et al., 1996b). In the coxa, three clusters of sensory cells associated with the hair plates are stained close to the junction with the thorax. The number of stained cell bodies equals the number of observed sensory hairs composing the hair plate. In the trochanter, five cell clusters are detectable: three of them belong to hair plates, and the remainder to campaniform sensilla. In the femur, two clusters of sensory cells associated with campaniform sensilla are detected on the dorsal and ventral proximal surfaces. In addition, the chordotonal organs are stained as obvious structures. In the tibia, there are a pair of campaniform sensilla on the dorsal surface and three single campaniform sensilla on the ventral surface. The cell bodies corresponding to these sensilla are stained. In this segment, the structure that seems to correspond to the stretch receptor is also strongly stained close to the joint with the tasus. The tibia and tarsi bear a number of taste bristles accompanied by stained cell bodies in clusters. In most cases, four stained cells are recognizable in a cluster (Fig. 3). The taste bristles on the legs are multimodal sensory sensilla having usually four chemosensitive and a mechanosensitive neuron (Stocker, 1994). On the distal surface of the first, third, and fifth tarsomere, a pair of campaniform sensilla are present (Fig. 2A, CSs marked with asterisk; Merrit and Murphey, 1992). There are many stained cell bodies in each tarsomere, including the neurons of the taste bristles. It is not yet known, however, which stained cell bodies belong to those campaniform sensilla (Yasuyama, unpublished results).

Wing. In the wing blade, mechanosensory and chemosensory bristles are located on the anterior wing margin (the first longitudinal vein, L1; Hartenstein and Posakony, 1989; Palka et al., 1979). A thin fiber bundle stained with X-gal is observed along L1 (Kitamoto et al., 1995). This bundle is likely to be the axon fibers from the neurons associated with chemosensory bristles. The sensory cells associated with the twined campaniform sensilla on vein L1, and the sensillum on the anterior cross vein (Huang et al., 1991) are obviously stained (Fig. 4A). The cells of the campaniform sensilla lying along vein L3 are stained only faintly (Yasuyama, unpublished results). In the distal radius vein, the cells associated with the dorsal and ventral humeral cross-vein sensilla, and the cell projecting a fiber into the giant sensillum of the radius (Cole and Palka, 1982) are stained (Fig. 4D–F). In the proximal and medial radius, where small campaniform sensilla occur in cluster (Cole and Palka, 1982), the stained cell bodies are found

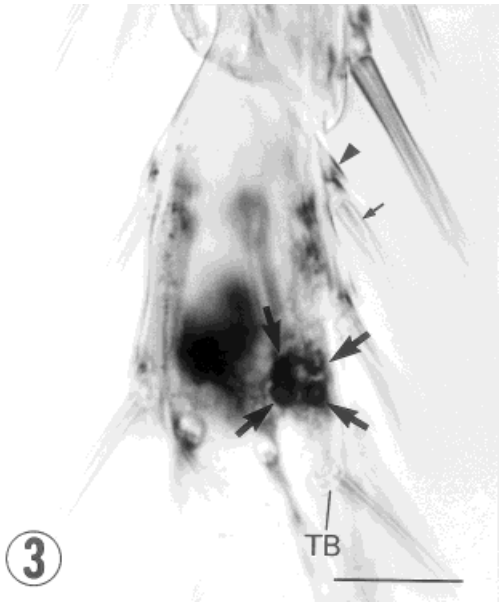

Fig. 3. X-gal staining of cell bodies associated with the taste bristle (TB) on a tarsomere of an adult prothoracic leg in 7.4 kb-ChAT/*lacZ* transformants. Four stained cell bodies (arrows) are recognizable in a cluster. The tactile bristle (thin arrow) is accompanied by a bract (arrowhead). The sensory cells of tactile bristles are not stained. Scale bar = 20 μm.

in a cluster (Fig. 4B,C). The sensory cells associated with the tegula sensilla are also stained (Yasuyama, unpublished results).

Anal Plates and Genitalia. Bristles of varying sizes and shapes occur in a distinct pattern on the surface of the anal plates and the male and female external genitalia (Taylor, 1989b). In the anal plates, which bear thin and curved bristles, X-gal staining is restricted to the cells under the particular bristles that are located on the ventral part of the anal plate. This staining pattern is shown in both the male and female (Kitamoto et al., 1995). In most cases, only a single cell is stained corresponding to each bristle. No stained cells are found associated with the bristles on the male genital arch and on the eighth tergite (Yasuyama, unpublished results). In the male genitalia, most of the bristles are thought to be mechanosensory, based on their external aspect (Taylor, 1989a). In the clasper of male genitalia, X-gal staining is observed in the cells in the clusters associated with a group of ventrally located, stout bristles. Staining is also observed at the base of the particular hypandrial bristles (Kitamoto et al., 1995). In the female genitalia, the three pairs of microbristles (sensilla trichodea) on the vaginal plate have been surmised to be chemosensory (Stocker, 1994; Taylor, 1989a). The uterus, following the vaginal plate, is strongly stained. This staining is probably due to endogenous X-gal hydrolyzing activity and presents an obstacle to further characterization of the sensory neurons associated with the bristles on the vaginal plate using the *lacZ* reporter gene approach (Yasuyama, unpublished results).

Buchner et al. (1993) have found in *Drosophila* that most sensory bristles including multimodal and tactile ones are associated with only one histamine-like immunoreactive neuron, and that campaniform sensilla or chordotonal organs do not stain. Based on these observations, they have suggested that the sensory cells of tactile bristles and the mechanosensory neurons of multimodal taste bristles are histaminergic. The observation that β-galactosidase reporting, using regulatory sequences from the ChAT gene, is restricted to the multimodal taste bristle and proprioceptors such as campaniform sensilla, is consistent with the above hypothesis. In locust, the femoral chordotonal organs and external proprioceptors have been shown to be cholinergic by their ChAT immunoreactivity (Lutz and Tyrer, 1988). Information about the transmitters used in chemosensory systems of arthropods is still rather limited. In *Manduca sexta*, antennal olfactory sensory neurons have been proposed to be cholinergic in addition to antennal mechanosensory neurons based on their AChE activity (Homberg et al., 1995; Stengl et al., 1990). In the decapode crustacea, immunocytochemical studies have shown that FMRFamide may serve as a transmitter of non-olfactory chemosensory neurons (Schmidt 1997; Schmidt and Ache, 1994).

DISTRIBUTION OF CHAT EXPRESSING NEURONS IN THE CENTRAL NERVOUS SYSTEM

Embryos

ChAT mRNA and enzymatic activity varies as a function of the developmental stage of *Drosophila*. ChAT mRNA is first detected approximately 6–7 hours after oviposition, increases through the first and second larval instar, decreases into early pupal stages, and increases again during late pupation, reaching a maximum in adults. The enzyme activity during development shows a similar qualitative pattern but exhibits a temporal scale that lags behind the mRNA changes by several hours. ChAT activity is first detected at nearly the same time as ChAT mRNA (6–7 hours after oviposition), increases through the first and second larval instar stages, reaches a maximum in the third instar, decreases throughout early pupation, then increases and reaches a second maximum between late pupal stages and young adults (Carbini et al., 1990).

In stage-15 embryos (12–14 hours after oviposition), in situ hybridization signals are detected broadly in the supraoesophageal ganglion. In each half segment of the ventral nerve cord, two clusters of cells express ChAT mRNA. The lateral cluster consists of about ten cells. The medial cluster is subdivided into two groups: the anterior cluster containing three cells, and the posterior cluster containing three or four cells (Yasuyama et al., 1995b). β-Galactosidase expression in stage-15 embryos of 7.4 kb/*lacZ* transformants is also observed in embryonic central nervous system. In the ventral nerve cord, however, fewer cells expressing reporter genes are detected in each segment compared with the number of cells revealed by in situ hybridization. Only a pair of cells located on each side of the midline, and a cluster laterally positioned to each of the paired cells is observed (Kitamoto and Salvaterra, 1993). ChAT immunoreactivity is first observed 16–18 hours after oviposition. Immunoreactivity is primarily localized to the neuropil region, and also to a small number of cell bodies in the cortices associated with the suboesopha-

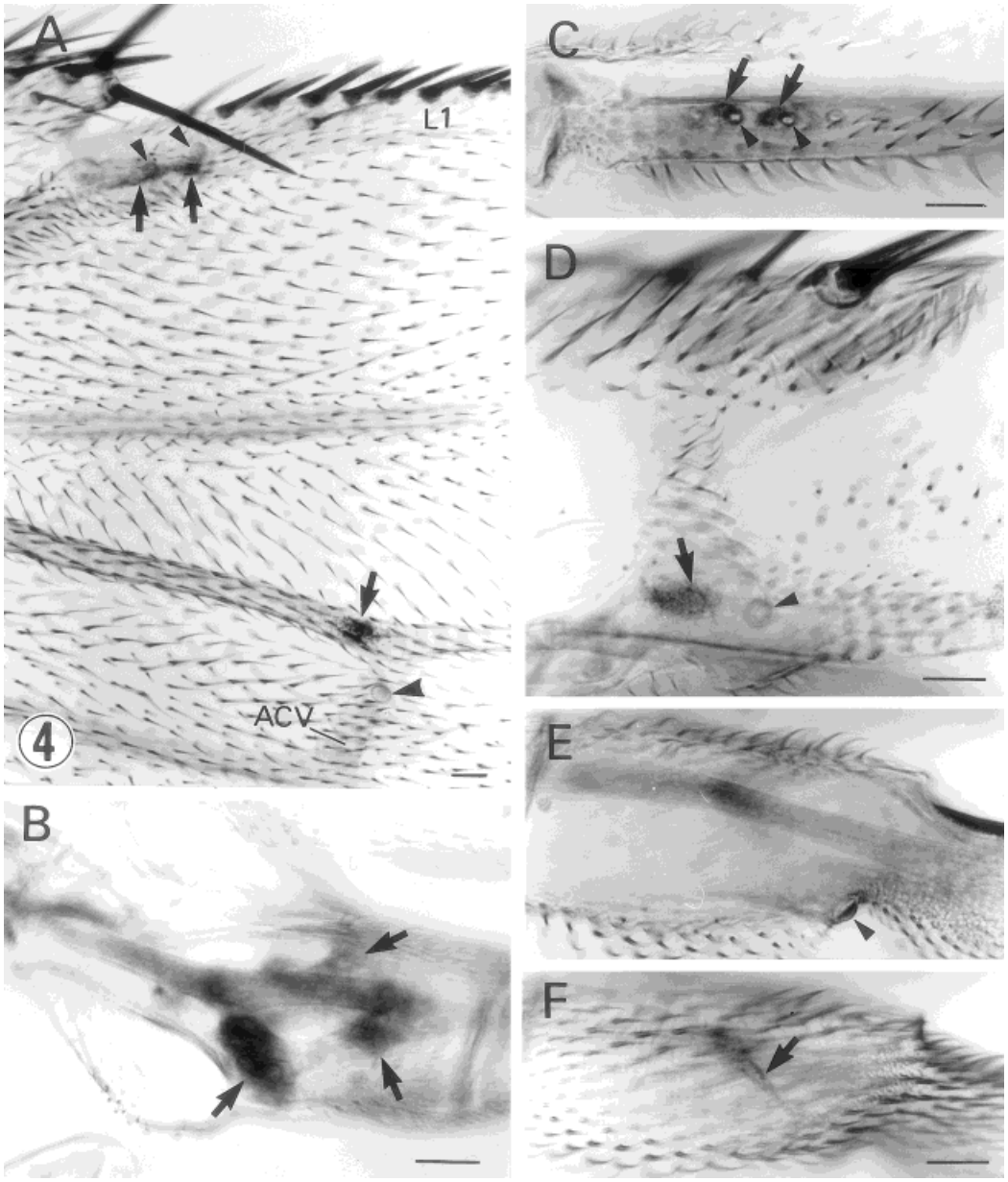

Fig. 4. Distribution of X-gal staining in the wing of adult 7.4 kb-ChAT/*lacZ* transformants. **A:** The stained cell bodies (arrows) associated with the twined campaniform sensilla (small arrowheads) on the first longitudinal diatal vein (L1), and with the campaniform sensillum on the anterior cross vein (ACV). **B:** The cell bodies associated with the clusters of small campaniform sensilla on dorsal surface of the proximal radius, which are strongly stained in clusters (arrows). **C:** The campaniform sensilla (arrowheads) on the ventral surface of medial radius and their stained cell bodies (arrows). D–F: Sensilla and stained cell bodies in the distal radius. **D:** The dorsal humeral cross-vein sensillum (arrowhead) and its large sensory cell (arrow). **E, F:** The giant sensillum of the radius (arrowhead in E) and its large sensory cell (arrow in F). E and F show the same field at different focal planes. Scale bars = 20 μm.

geal ganglion. The intensity of staining increases in the more fully developed central nervous system of embryos 2–4 hours before hatching (Gorczyca and Hall, 1987).

Larvae

Brain. The pattern of ChAT immunoreactivity in first instar larvae is very similar to that of late embryo. In second larval instar, the brain lobes are readily discernible, and the calyces of the mushroom bodies are apparently stained (Gorczyca and Hall, 1987). In late third instar larvae, the calyces are prominently stained as a spherical structure at the top of the neuropil region (Yasuyama et al., 1995b). In the anteromedial region of the protocerebrum, four to six cell bodies exhibit strong ChAT immunoreactivity (Gorczyca and Hall, 1987; Yasuyama et al., 1995b). These cells are likely to be identical to the median neurosecretory cells within the pars intercerebralis of adult brain. In situ hybridization signal for ChAT mRNA, β-galactosidase expression and

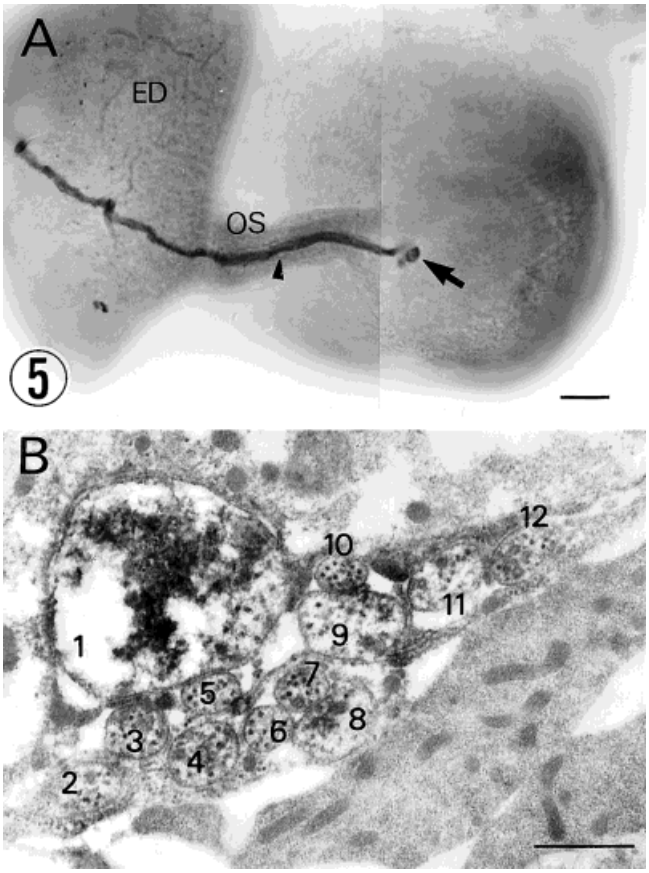

Fig. 5. ChAT immunoreactivity in Bolwig's nerve and ChaLV neuron of a late third instar larva. **A:** A whole-mount preparation showing a strongly stained ChaLV cell bodies (arrow), which is located near the insertion site of the optic stalk (OS), and Bolwig's nerve (arrowhead), which runs on the surface of eye imaginal disc (ED) and optic stalk. **B:** An electron micrograph of a transverse section of Bolwig's nerve running through the optic stalk. All twelve axons (1–12) composing Bolwig's nerve show apparent ChAT immunoreactivity. Scale bar in A = 20 μm; in B = 1 μm.

ChAT immunoreactivity are all detected in the corresponding cells of larval, pupal, and adult brain (Yasuyama et al., 1995b). In locust, the neurosecretory cells of the pars intercerebraris in adult brain also show acetylcholine and ChAT immunoreactivity (Geffard et al., 1985). In addition, a monopolar cell that is located proximal to the larval optic neuropil and terminates its axon in the suboesophageal neuropil is apparent (Gorczyca and Hall, 1987). Many β-galactosidase expressing cell bodies are detected in the cortices associated with the larval central brain of the late third instar larvae (Kitamoto and Salvaterra, 1993). This suggests that a large number of cholinergic neurons are involved in the ChAT immunoreactivity found in the neuropil.

In the larval optic lobe, Bolwig's nerve (Gorczyca and Hall, 1987; Yasuyama et al., 1995b) as well as a neuron close to the insertion site of the optic stalk (referred to as ChaLV; Yasuyama et al., 1995b) exhibit ChAT immunoreactivity (Fig. 5A). A projection of ChaLV runs in parallel with Bolwig's nerve to the larval optic visual neuropil. This neuron is likely to be one of the larval first order visual interneurons described as optic lobe pioneers due to the similarity in cell body position and axon trajectory (Tix et al., 1989). Bolwig's nerve, running on the surface of the optic stalk, consists of twelve fibers showing ChAT immunoreactivity (Fig. 5B), suggesting that all twelve of the larval photoreceptors comprising Bolwig's organs are cholinergic. β-Galactosidase expression is also detected in the neurons corresponding to ChaLV in the larval and early pupal optic lobe, as well as in Bolwig's organ and its nerve (Yasuyama et al., 1995b).

Ventral Ganglia. The larval ventral ganglia consist of fused suboesophageal, thoracic and abdominal ganglia. In the ventral ganglia, as in the brain, ChAT immunoreactivity is widely distributed throughout almost the entire neuropil, except the specific structures in the thoracic neuromeres (thin arrows in Fig. 6B). Each of the thoracic neuromeres contains a pair of conspicuous ventro-lateral swellings of neuropil with a finely textured aspect, which seem to correspond to the imaginal leg neuropil described by Merritt and Murphey (1992). These neuropils are not stained exclusively. The commissures with immunoreactive fibers are seen in a segmentally repeated array. The stained commissures are distinct in the suboesophageal and thoracic ganglia (Fig. 6A). Immunolabled fibers are also seen running through the cortices to the neuropil regions (thick arrow in Fig. 6B). These fibers may be the axons originating from larval sensory cells. Segmentally arranged, bilaterally paired cell bodies are stained in the ventro-lateral cortices associated with the thoracic neuromeres (Fig. 6C).

Pupae

ChAT immunoreactivity in ChaLV is detectable in its axon but not in cell bodies until about 60 hours after puparium formation (APF) (arrowheads in Fig. 7A; Yasuyama et al., 1995b). Thereafter, no staining of ChaLV or its axon can be observed. β-Galactosidase activity, in contrast, is detected in the ChaLV cell body of 40-hour APF pupae. The discrepancy between the results of these two different but complementary techniques may be attributed to the stability of β-galactosidase relative to ChAT protein. ChAT immunoreactivity in Bolwig's nerve is not detected in the optic lobe of 24-hour APF pupae (Yasuyama et al., 1995b).

ChAT immunoreactivity in the developing brain noticeably decreases by 36-hour APF (Fig. 7A), and disappears almost entirely by 60 hours. Thereafter, the immunoreactivity increases gradually, in conformity with the development of brain and optic lobes (Gorczyca and Hall, 1987). In the optic lobes of 70-hour APF pupae, three faintly stained layers are discernible in the medulla (Fig. 7B). In the lobula and lobula plate, only very faint labeling is detected. In the pupae of 85-hour APF, four layers in the lobula are apparently stained, in addition to three layers in the medulla (Fig. 7C). In the lobula plate, four well-stained layers are detected. This contrasts with newly eclosed adults where the posterior two layers of the lobula plate show apparent ChAT immunoreactivity, while the anterior two do not (Fig. 7D). A similar staining pattern in the lobula plate is observed in mature adults (Yasuyama et al., 1995a). These observations suggest that negative regulation of the ChAT gene suppresses ChAT expres-

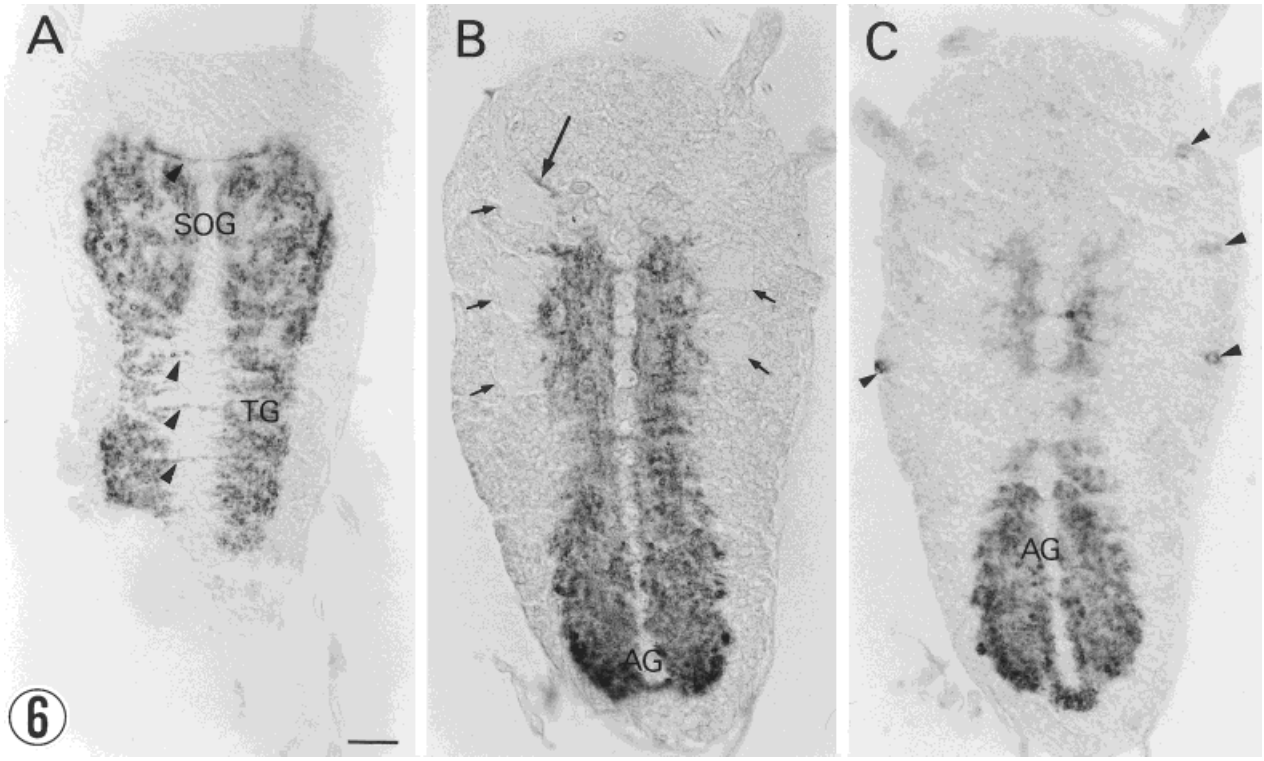

Fig. 6. ChAT immunoreactivity in the larval ventral ganglia. Horizontal sections at different levels from dorsal (A) to ventral (C). Staining is observed in nearly the entire neuropil of the ganglia. **A:** The commissures (arrowheads) in the suboesophageal (SOG) and thoracic (TG) neuromeres show immunoreactivity as well as the neruopils. **B:** Paired conspicuous ventro-lateral neuropils (thin arrows) in each thoracic neuromere are not stained. A fiber running through the cortices of the pro-thoracic neuromere (thick arrow) indicates immunoreactivity. **C:** Bilaterally paired cell bodies in the thoracic neuromeres (arrowheads) are stained in the ventro-lateral cortices. AG, abdominal ganglia. Scale bar = 20 μm.

sion in the anterior two layers of lobula plate just before or after eclosion.

Adults

Optic Lobe. In the lamina of some preparations, the optic cartridge and radially arranged dot-like structures proximal to the lamina cell body layer show ChAT immunoreactivity (Fig. 7D,E; Buchner et al., 1986; Gorczyca and Hall, 1987; Ikeda and Salvaterra, 1989; Yasuyama et al., 1995a). The stained structures in the distal lamina seem to be the synaptic terminals for intrinsic or centripetally projecting neurons (Buchner et al., 1986; Gorczyca and Hall, 1987). Buchner et al. (1986) have pointed out the similarity of this structure to the terminals of the C2 interneurons. More recent evidence that C2 interneurons in the blowfly are GABAergic (Datum et al., 1986; Meyer et al., 1986) make this less likely. Therefore, the identity of the ChAT immunoreactive lamina structure should be considered as unresolved (Buchner et al., 1988). In the medulla, three to five or more layers are distinctly stained (Fig. 7D; Buchner et al., 1986; Gorczyca and Hall, 1987; Ikeda and Salvaterra, 1989; Yasuyama et al., 1995a). The distal two or three stained layers have been suggested to correspond to the layers of the synaptic terminals from L1 and L2 laminar monopolar neurons (Ikeda and Salvaterra, 1989). Laminar monopolar neuron L4 and L5 also terminate in the second layer proximal to the outermost one (Fischbach and Dittrich, 1989). This does not exclude the possibility that these neurons contribute to ChAT immunostaining in the medulla. Figure 7E shows two types of ChAT immunoreactive cell bodies in lamina cortex, the large ones lying distally and the small ones lying proximally. The terminals of the medulla T neurons have been suggested as candidates for staining of the innermost layer proximal to the serpentine layer (Ikeda and Salvaterra, 1989). In the lobula, four distinct immunoreactive layers can be seen. In the lobula plate, the immunolabeling is diffuse (Buchner et al., 1986), or two layers are recognized in the posterior half (Yasuyama et al., 1995a).

Except for the lamina monopolar neurons, the cell bodies in the optic lobe are not distinctly immunolabeled in general, whereas in situ hybridization with a radioactive (Barber et al., 1989) or a nonradioactive (Yasuyama et al., 1996a) cRNA probe specific for *Drosophila* ChAT mRNA and X-gal staining of 7.4kb-ChAT/*lacZ* transformants (Kitamoto et al., 1992) determined the location of cell bodies expressing the ChAT gene in the optic lobe cortices. These latter two techniques reveal heavily labeled cell bodies in the lamina cell body layer, in the cortices on both the anterior and posterior sides of the first chiasm, and in the cortices located anterior to medulla and posterior to the lobula plate (Kitamoto et al., 1992; Yasuyama et al., 1996a). These

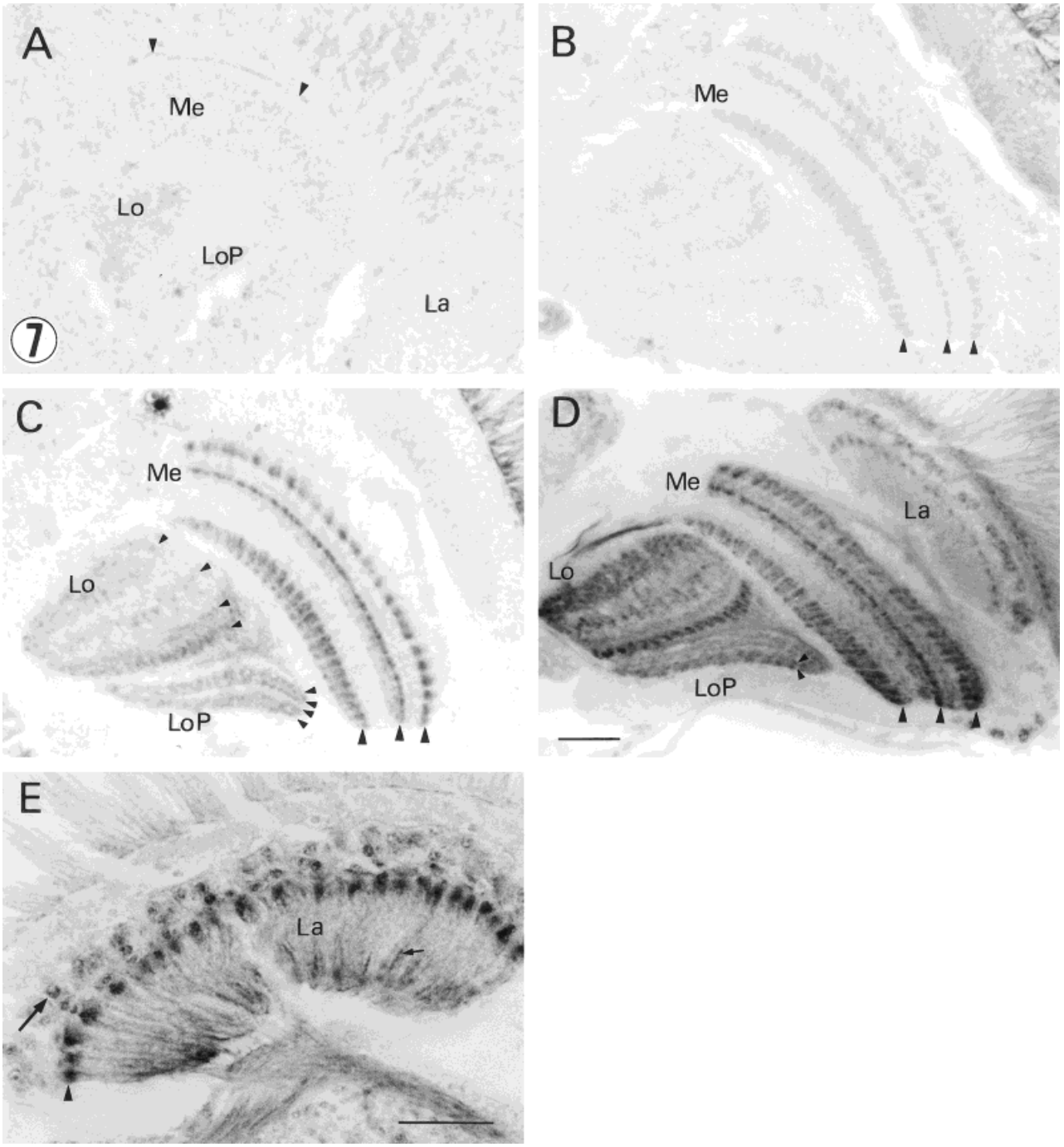

Fig. 7. ChAT immunoreactivity in horizontal sections of pupal and adult optic lobes. **A:** A 30-hour APF pupa. The immunoreactive axon of the ChaLV (arrowheads) runs along the distal edge of the developing medulla (Me). **B:** A 70-hour APF pupa. Three faintly stained layers (arrowheads) are seen in the medulla (Me). **C:** An 85-hour APF pupa. In the lobula (Lo) and lobula plate (LoP), four stained layers are detectable (small arrowheads), respectively, in addition to three layers (large arrowheads) in the medulla. **D:** A freshly eclosed adult. The immunoreactive three (large arrowheads) and four layers (small arrowheads) are apparent in the medulla and lobula, respectively. In the lobula plate, posterior two layers (arrowheads) show stronger immunoreactivity compared to the anterior two. Note that cell bodies (thick arrow) in the lamina cortex, and dot-like structure (thin arrow) in the distal region of the lamina (La) are stained. **E:** A horizontal section of the adult lamina. In the lamina cartridge (La), the radially arranged dot-like structures (arrowhead) in the distal region and the fine fibrous structures (thin arrow) in the proximal half are stained distinctively. In this preparation, the cell bodies of lamina neurons are also stained (thick arrow). Scale bar = 20 μm.

observations are consistent with the suggestion, based on ChAT immunocytochemical observations, that some of the laminar monopolar neurons and medullar T neurons may be cholinergic.

Brain. In the cephalic and the suboesophageal ganglion, rather strong ChAT immunoreactivity is widely detected throughout almost the entire neuropil (Fig. 8), with the exception of the three lobes and peduncles of the mushroom bodies, which are nearly completely unstained (Fig. 8A). The antennal lobes (Figs. 8A, 9B), the antennal mechanosensory projections (Fig. 8B), the calyces of the mushroom bodies (Fig. 8D), and the optic tubercles (Fig. 8A) are prominently labeled (Buchner et al., 1986; Gorczyca and Hall, 1987; Yasuyama et al., 1995a). Some other neuropil regions, the lateral tip of the accessory protocerebrum (Fig. 8A) and the lateral protuberance of ventral protocerebrum (Fig. 8B), are also strongly stained. Among the neuropils associated with the central complex, the caudal half of the ellipsoid body, the noduli, and the lateral triangles are strongly stained, while the fan-shaped body and the protocerebral brides are moderately stained (Figs. 8B, 9A; Buchner et al., 1986; Yasuyama et al., 1995a). In addition, strong immunoreactivity is detected in some fiber bundles in the ganglia: the ocellar nerve, which consists of six to eight distinctively stained axons with large diameters terminating in the posterior slope with extensive arborization (Fig. 8D,F; Yasuyama et al., 1995a), the antennal glomerular tract (Fig. 8D; Buchner et al., 1986; Gorczyca and Hall, 1987; Yasuyama et al., 1995a), the tract connecting the lateral protocerebrum with the ipsilateral deutocerebrum (Fig. 8C), the commissure connecting the right and left deutocerebrum (Fig. 8D), and the commissure in the subesophageal ganglion (Fig. 8E). In contrast, the commissures just anterior and posterior to the noduli of the central complex exhibit no labeling (Yasuyama et al., 1995a).

In the cephalic ganglion, in situ hybridization with a nonradioactive cRNA probe revealed a number of cell bodies expressing ChAT transcripts in almost the entire cortical region (Yasuyama et al., 1996a), including the antennal lobe cortex where ChAT immunoreactive cell bodies are detected (Fig. 9B; Gorczyca and Hall, 1987). The cell bodies in the cortices anterior to or between the right and left antennal lobes, as well as the cortical regions lateral to the antennal lobe, exhibit substantial amounts of ChAT mRNA. Some of these cells are probably the source of immunoreactivity in the antennal glomerular tracts, which carry the axons of antennal relay interneurons (Yasuyama et al., 1996a), suggesting that some of the antennal interneurons are cholinergic. X-gal staining of the 7.4 kb/*lacZ* transformants also reveals cell bodies expressing reporter gene in the antennal lobe cortex (Kitamoto et al., 1992). In the suboesophageal ganglion, ChAT mRNA-expressing cell bodies are detected in the lateral cortical region. Strongly labeled cells are also found along the midline on the anteroventral cortex situated posterior to the sucking pump (Yasuyama et al., 1996a). These neurons are suggested to resemble the giant symmetric relay interneurons described by Stocker et al. (1990) (Yasuyama et al., 1996a). Two sets of neurons are detected by their distinctive staining with the different but complementary techniques for detecting the cholinergic neurons: the median neurosecretory cells in the pars intercerebralis (see also Brain), and two groups of paired neurons in the posterior cortical rind designated as PPM neurons located in the median region dorsal to the esophagus, and PPL neurons located laterally to the esophagus (Yasuyama et al., 1996a). These neurons are characterized by the persistence of ChAT mRNA expression and ChAT immunoreactivity in *Cha* temperature-sensitive mutants exposed to a restrictive temperature. In contrast, most other cholinergic neurons in the mutants down-regulate *Cha* gene expression at restrictive temperature. In these specific cholinergic neurons, ChAT expression may thus be controlled in a manner that is different from other cholinergic neurons (Yasuyama et al., 1996a). The functions and entire structures of the PPL and PPM neurons remain to be resolved. A variety of peptidergic phenotypes have been described for the median neurosecretory cells of *Drosophila* and larger flies by immunocytochemistry (Duve and Thorpe, 1979, 1981, 1983; Nässel and O'Shea, 1987; O'Brien et al., 1991; White et al., 1986), and in some cells, co-localization of two different neuropeptides has been demonstrated (Nässel, 1993). If the cholinergic median neurosecretory cells of *Drosophila* pars intercerebralis also contain other neuropeptides, such as FMRFamide, these cells may provide a useful model system to investigate molecular mechanisms regulating production of co-transmitters.

Thoracico-abdominal Center. The thoracic-abdominal center is composed of fused thoracic and abdominal ganglia (Power, 1948). The tectulum, which overlays the segmental neuromeres (Power, 1948), is almost uniformly stained with a coarse granular aspect by anti-ChAT antiserum (Fig. 10C–E). Each of the thoracic neuromeres has a pair of leg neuropils, which are ventro-lateral swellings of neuropil associated with leg motor and sensory function (Merritt and Murphey, 1992). The leg neuropils, in contrast to the tectulum, display ChAT immunoreactivity with a fine granular aspect (Fig.10A–C). Distinct staining is seen in the anterior and posterior edge of the anterior and posterior lateral association center, respectively, and in the region along the oblique tract, and also in the ventromedial region of the lateral ventral association center, in each leg neuropil (Fig. 10). The medial ventral association center (mVAC) in each thoracic neuromere (Fig. 10A,C, and E) and the dorsal and ventral longitudinal tracts are also stained distinctively (Fig. 10A,B, and E). The ChAT immunoreactive longitudinal tracts may correspond to the faciculi described by Power (1948), that is, the haltere nerves, the median tract of dorsal cervical fasciculus, the intermediate tract of dorsal fasciculus, and the dorsal lateral tract of ventral fasciculus. Further studies, however, are required to precisely characterize the ChAT immunoreactive fasciculi found in the thoracico-abdominal center. The ventral neuropil region within the accessory mesothoracic neuromere (Power, 1948), where the sensory neurons in the wings mainly terminate, termed "ovoid" (Palka et al., 1979) also exhibits ChAT immunoreactivity (arrows in Fig. 10D). In the abdominal ganglia, staining is seen in the entire neuropil region. The commissures with immunoreactive fibers are observed indistinctly in a segmentally repeated array (Yasuyama, unpublished results).

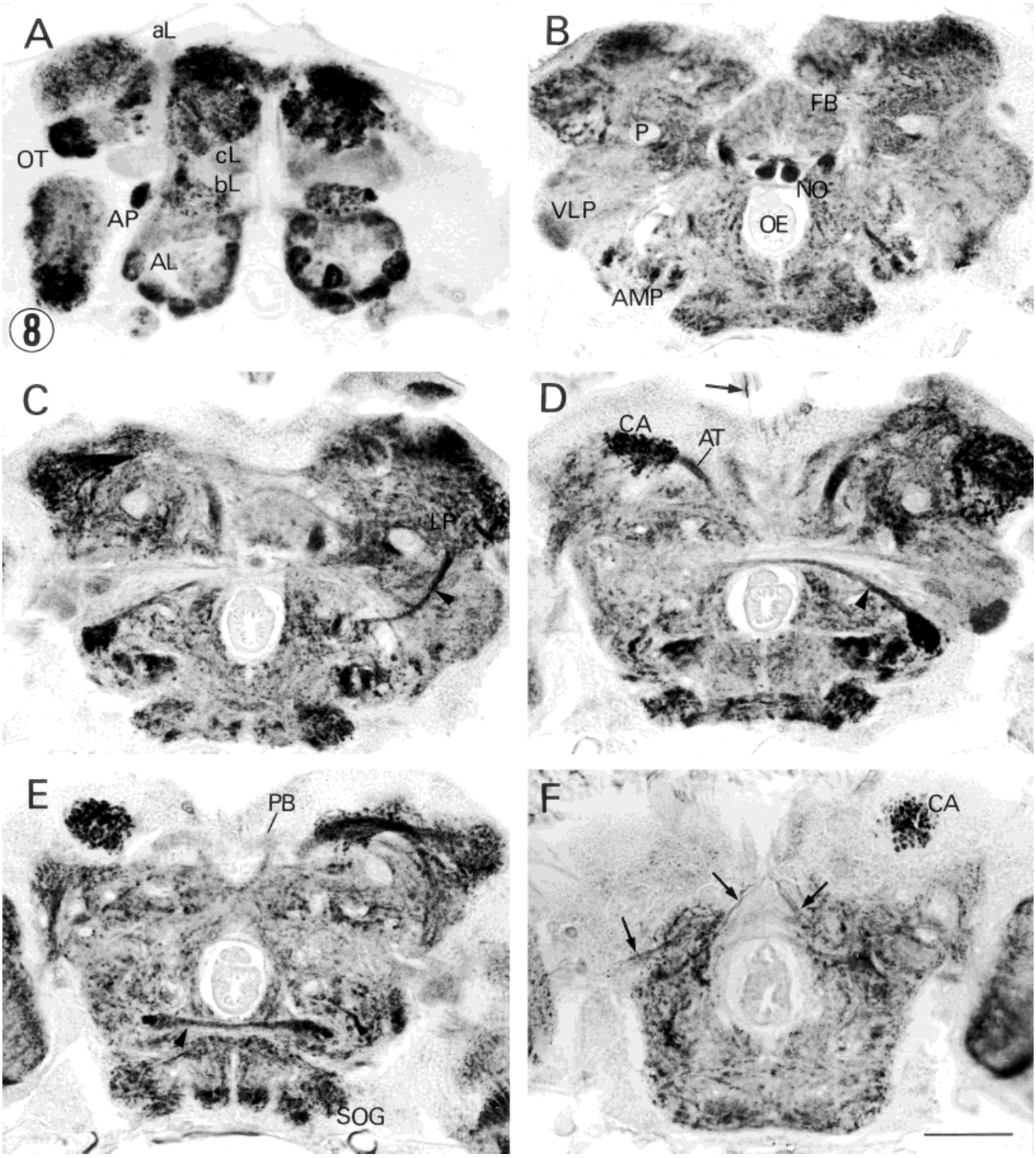

Fig. 8. ChAT immunoreactivity in the adult brain. A–F: Frontal sections are shown at various levels of brain corresponding to anterior-to-posterior sections. Staining is observed in almost the entire neuropil. Notable unstained regions include the three lobes (alpha lobe, aL; beta lobe, bL; gamma lobe, cL; **A**) and the peduncle (P) of the mushroom bodies (**B**). Arrowheads in **C** and **D** indicate the nerve tract connecting the lateral protocerebrum (LP) with deutocerebrum and the commissure connecting right and left deutocerebrum, respectively. Arrows in D and **F** indicate the axons of ChAT immunoreactive ocellar interneurons. Arrowhead in **E** shows the commissure in the subesophageal ganglion (SOG). AL, antennal lobe; AMP, antennal mechanosensory projection; AP, lateral tip of accessory protocerebral lobe; AT, antennal glomerular tract; CA, calyces; FB, fan-shaped body; NO, noduli; OE, esophagus; OT, optic tubercle; PB, protocerebral bridge, VLP, ventrolateral protocerebrum. Scale bar = 50 μm.

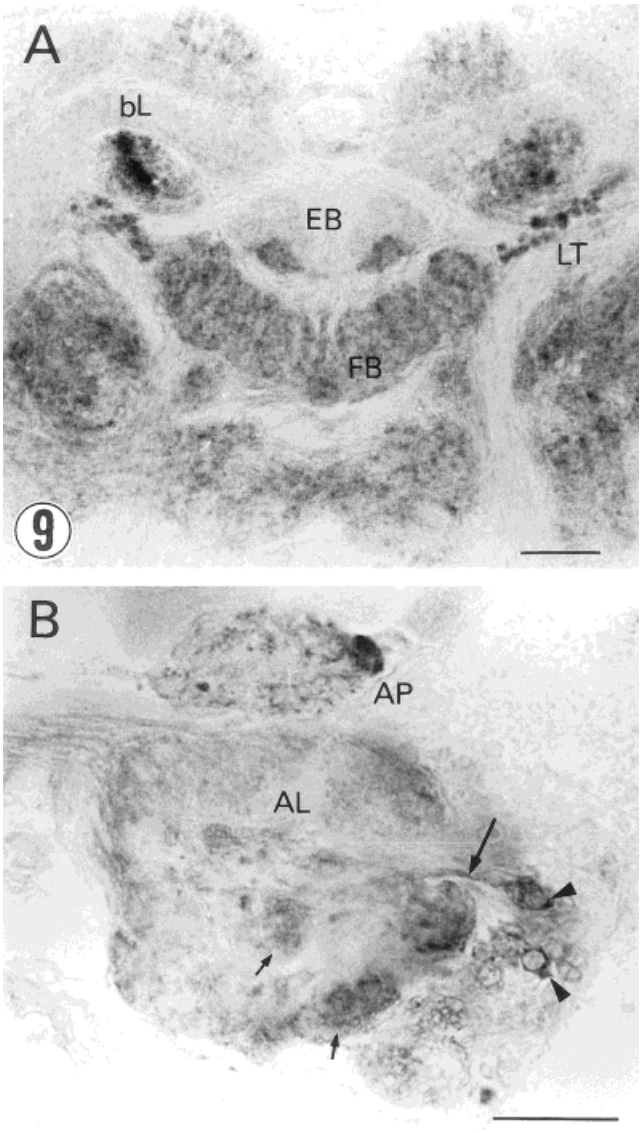

Fig. 9. ChAT immunoreactivity in the adult brain. **A:** A horizontal section of the central complex. The posterior half of the ellipsoid body (EB) and the lateral triangles (LT) exhibit strong immunoreactivity. The fan-shaped body (FB) is also stained. The lobes of mushroom body (bL) are not stained. **B:** A frontal section of the antennal lobe (AL). The glomeruli (thin arrows) are stained individually. Stained cell bodies (arrowheads) are seen in the lateral cortex. An immunoreactive axon (thick arrow) from a stained cell body projects into the antennal lobe. The lateral tip of accessory protocerebral lobe (AP) is also strongly stained. Es, esophagus. Scale bars = 20 μm.

In flies, modality-specific segregation of afferent axonal arbors, originating from the leg sensory organs, in the thoracic neuromere has been demonstrated by Murphey et al. (1989). The chemosensory neurons associated with taste bristles of legs project to the ventro-medial neuropil regions of each leg neuropil, corresponding to the ChAT immunoreactive regions (Fig.10). The afferent axons of the proprioceptors such as hair plates and campaniform sensilla terminate in the anterior and posterior lateral association center, and in the neuropil region along the oblique tract of the leg neuropils (Merritt and Murphey, 1992; Smith and Shephard, 1996).

The axons of sensory neurons of the femoral chordotonal organs (FCO) run along the oblique tract and terminate in mVAC (Smith and Shephard, 1996). These facts and the distribution pattern of ChAT immunoreactivity suggest that the chemosensory and proprioceptive sensory neurons in the leg, including FCO neurons, and in the wing are cholinergic. This is also consistent with the observations that X-gal staining of the 7.4 kb/*lacZ* transformants shows reporter gene expression in chemosensory and proprioceptive sensory neurons including FCO in the legs (see also Adult, leg, and wing; Kitamoto et al., 1995; Yasuyama et al., 1996b).

PERSPECTIVES

The gene for the vesicular acetylcholine transporter (VAChT) has recently been identified in *Drosophila* and specific antibodies have been generated (Kitamoto et al., 1998). It will be interesting in future studies to localize VAChT expression and compare its distribution to ChAT since both genes form a cholinergic locus and appear to be under common transcriptional regulation. Electron microscopic immunocytochemistry using antisera against both ChAT and VAChT proteins will reveal the intracellular localization of these proteins, and identify cholinergic synapses in the nervous system. In addition to these antisera, specific antisera against the subunit proteins of *Drosophila* muscarinic (Blake et al., 1993) and nicotinic (Jonas et al., 1994; Schuster et al., 1993) receptors have also been raised. The lamina of the optic lobe might be a good model to investigate cholinergic transmission by immunocytochemical approaches using antibodies related to cholinergic macromolecules. ChAT immunocytochemistry implies that some of lamina neurons are cholinergic, and there is an excellent catalogue of the synaptic connections of all columnar elements in the lamina (Meinertzhagen and O'Neil, 1991).

The development of knowledge regarding cholinergic neuron distribution in *Drosophila*, coupled with the power of genetic analysis of this organism, is sure to result in new insights into neurotransmitter phenotype selection and maintenance. This knowledge will help formulate questions regarding the contribution of a particular neurotransmitter system to nervous system function. Future genetic, molecular, and physiological studies in *Drosophila* should provide a deeper understanding of how this organism makes and uses acetylcholine to generate particular behaviors and physiological functions. Finally, the nature of cholinergic deficits seen in a variety of human neurodegenerative diseases such as Alzheimer's disease and amyotrophic lateral sclerosis should benefit from our ability to understand cholinergic neurobiology in a model experimental organism such as *Drosophila*.

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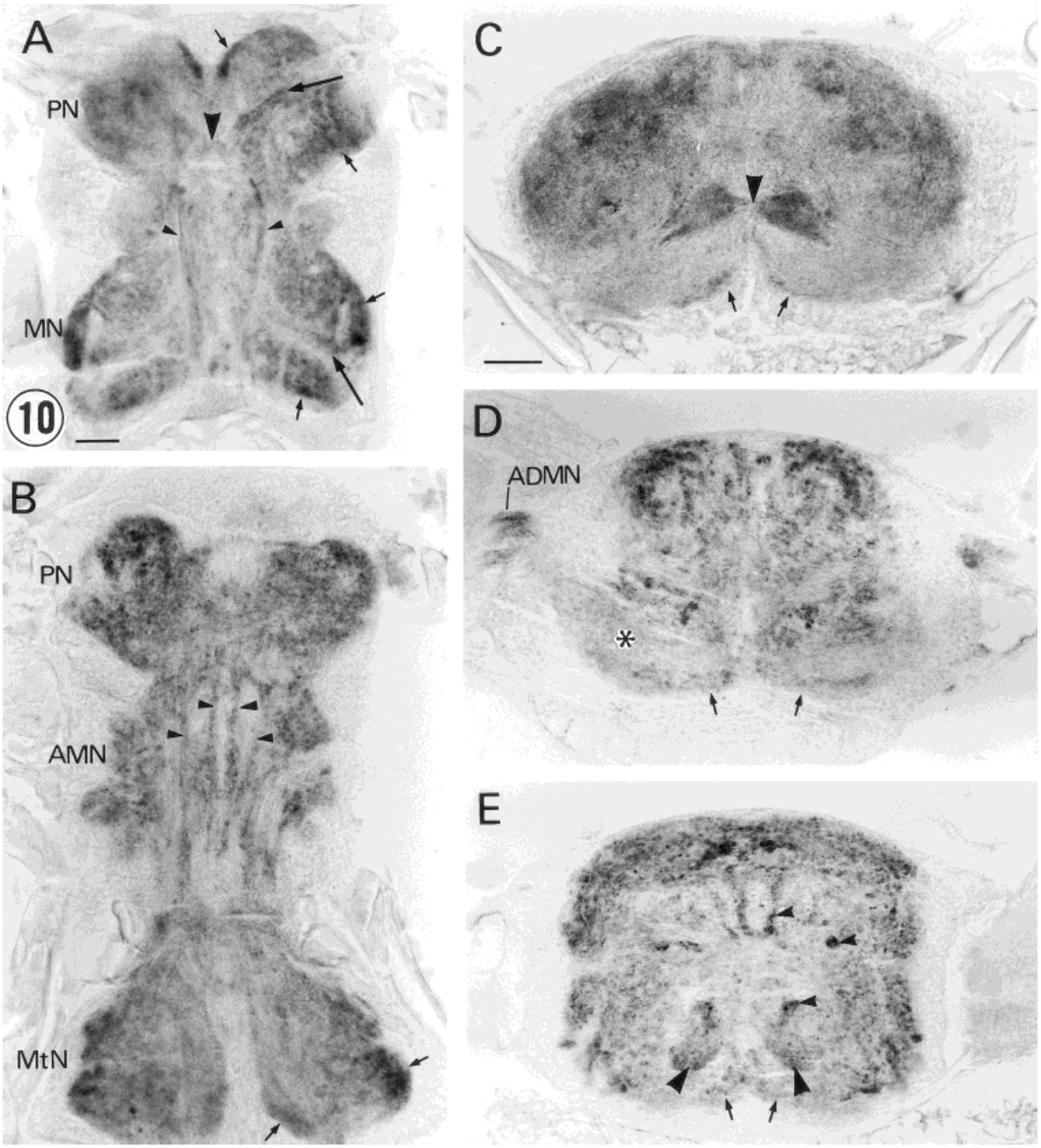

Fig. 10. ChAT immunoreactivity in the adult thoracico-abdominal center. **A,B:** Horizontal sections showing the pro- (PN), meso- (MN), and metathoracic (MtN) leg neuropils, and the accessory mesothoracic neuromere (AMN) at two different levels: A shows a more ventral level than that of B. Distinct staining is seen in the anterior and posterior edges (thin arrows) and in the median region (thick arrows) of each leg neuropil. The paired longitudinal axon tracts (small arrowheads) and mVAC (large arrowhead) also show immunoreactivity. **C–E:** Frontal sections at the level of prothoracic (C), accessory mesothoracic (D), and mesothoracic neuromere (E). In each neuromere, the ventro-medial regions (thin arrowheads) are distinguishable by their immunoreactivity. The mVAC in pro- (large arrowhead in C) and in mesothoracic neuromere (large arrowheads in E) is strongly stained. Paired cross profiles of the longitudinal axon tracts also exhibit strong immunoreactivity. Small arrowheads in E indicate the immunoreactive profiles detected in the half side of the mesothoracic neuromere. Asterisk in D shows the ovoid. ADMN, anterior dorsal mesothoracic nerve. Scale bars = 20 μm.

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REFERENCES

Barber RP, Sugihara H, Lee M, Vaughn JE, Salvaterra PM. 1989. Localization of *Drosophila* neurons that contain choline acetyltransferase messenger RNA: an in situ hybridization study. J Comp Neurol 180:533–543.

Blake AD, Anthony NM, Chen HH, Harrison JB, Nathanson NM, Sattelle DB. 1993. *Drosophila* nervous system muscarinic acetylcholine receptor: transient functional expression and localization by immunocytochemistry. Mol Pharmacol 44:716–724.

Bolwig N. 1946. Senses and sense organs of the anterior end of the house fly larvae. Vidensk Medd Dansk Naturh Foren 109:81–217.

Buchner E. 1991. Genes expressed in the adult brain of *Drosophila* and effects of their mutations on behavior: a survey of transmitter- and second messenger-related genes. J Neurogent 7:153–192.

Buchner E, Buchner S, Crawford G, Mason WT, Salvaterra PM, Sattelle DB. 1986. Choline acetyltransferase-like immunoreactivity in the brain of *Drosophila melanogaster*. Cell Tissue Res 246:57–62.

Buchner E, Bader R, Buchner S, Cox J, Emson PC, Flory E, Heinzmann CW, Hemm S, Hofbauer A, Oertel WH. 1988. Cell-specific immuno-probes for the brain of normal and mutant *Drosophila melanogaster*. I. Wild type visual system. Cell Tissue Res 253: 357–370.

Buchner E, Buchner S, Burg MG, Hofbauer A, Pak WL, Pollack I. 1993. Histamine is a major mechanosensory neurotransmitter candidate in *Drosophila melanogaster*. Cell Tissue Res 273:119–125.

Carbini LA, Maines VJM, Salvaterra PM. 1990. Developmental expression of choline acetyltransferase mRNA in *Drosophila*. Neurochem Res. 15:1089–1096.

Chase BA, Kankel DR. 1988. On the role of acetylcholine metabolism in the formation and maintenance of the *Drosophila* nervous system. Dev Biol 125:361–380.

Cole ES, Palka J. 1982. The pattern of campaniform sensilla on the wing and haltere of *Drosophila melanogaster* and several of its homeotic mutants. J Embryol Exp Morphol 71:41–61.

Crawford G, Slemmon JR, Salvaterra PM. 1982. Monoclonal antibodies selective for *Drosophila melanogaster* choline acetyltransferase. J Biol Chem 257:3853–3856.

Datum K-H, Weiler R, Zettler F. 1986. Immunocytochemical demonstration of -amino butyric acid and glutamic acid decarboxylase in R7 photoreceptors and C2 centrifugal fibers in the blowfly visual system. J Comp Physiol A 159:241–249.

Duve H, Thorpe A. 1979. Immunofluorescent localization of insulin-like material in the median neurosecretory cells of the blowfly C*alliphora vomitoria* (Diptera). Cell Tissue Res 200:187–191.

Duve H, Thorpe A. 1981. Gastrin/cholecystokinin (CCK)-like immunoreactive neurons in the brain of the blowfly C*alliphora erythrocephala* (Diptera). Gen Comp Endocrinol 43:381–391.

Duve H, Thorpe A. 1983. Immunocytochemical identification of -endorphin-like material in neurons of the brain and corpus cardiacum of the blowfly C*alliphora vomitoria* (Diptera). Cell Tissue Res 233: 415–426.

Ewing AW. 1978. The antenna as a "love song" receptor. Physiol Entomol 3:33–36.

Fischbach K-F, Dittrich APM. 1989. The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure. Cell Tissue Res 258:441–475.

Geffard M, Vieillemaringe J, Heinrich-Rock A-M, Duris P. 1985. Anti-acetylcholine antibodies and first immunocytochemical application in insect brain. Neurosci Lett 57:1–6.

Ghysen A, Dambly-Chaudiere C, Aceves E, Jan LY, Yan YN. 1986. Sensory neurons and peripheral pathways in *Drosophila* embryos. Roux's Arch Dev Biol 195:281–289.

Gorczyca MG, Hall JC. 1984. Identification of a cholinergic synapse in the giant fiber pathway of *Drosophila* using conditional mutations of acetylcholine synthesis. J Neurogenet 1:289–313.

Gorczyca M.G, Hall JC. 1987. Immunohistochemical localization of choline acetyltransferase during development and in Cha^{ts} of *Drosophila melanogaster*. J Neurosci 7:1361–1369.

Gorczyca MG, Budnik V, White K, Wu CF. 1991. Dual muscarinic and nicotinic action on a motor program in *Drosophila*. J Neurobiol 22:391–404.

Greenspan RJ. 1980. Mutations of choline acetyltransferase and associated neural defects in *Drosophila melanogaster*. J Comp Physiol 137:83–92.

Gundelfinger ED. 1992. How complex is the nicotinic receptor system of insect? Trends Neurosci 15:206–211.

Gundelfinger ED, Hess N. 1992. Nicotinic acetylcholine receptors of the central nervous system of *Drosophila*. Biochim Biophys Acta 1137:299–308.

Hall JC, Kankel DR. 1976. Genetics of acetylcholinesterase in *Drosophila melanogaster*. Gentics 83:517–535.

Hall LMC, Spierer P. 1986. The *Ace* locus of *Drosophila melanogaster*: structural gene for acetylcholinesterase with an usual 5' leader. EMBO J 5:2949–2954.

Hannan F, Hall LM. 1993. Muscarinic acetylcholine receptors in invertebrates: comparisons with homologous receptors from vertebrates. In: Pichon Y, editor. Comparative molecular neurobiology. Basel: Birkhauser Verlag; p 98–145.

Hartenstein V, Posakony JW. 1989. Development of adult sensilla on the wing and notum of *Drosophila melanogaster*. Development 107:389–405.

Homberg U, Hoskins SG, Hildebrand JG. 1995. Distribution of acetylcholinesterase activity in the deutocerebrum of the sphinx moth *Manduca sexta*. Cell Tissue Res 279:249–259.

Huang F, Dambly-Chaudiere C, Ghysen A. 1991. The emergence of sense organs in the wing disc of *Drosophila*. Development 111:1087–1095.

Ikeda K, Salvaterra PM. 1989. Immunocytochemical study of a temperature-sensitive choline acetyltransferase mutant of *Drosophila melanogaster*. J Comp Neurol 280:283–290.

Itoh N, Slemmon J R, Hawke DH, Williamson R, Morita E, Itakura K, Roberts E, Shively JE, Crawford GD, Salvaterra PM. 1986. Cloning of *Drosophila* choline acetyltransferase cDNA. Proc Natl Acad Sci USA 83:4081–4085.

Jan YN, Ghysen A, Christoph I, Barbel S, Jan LY. 1985. Formation of neuronal pathways in the imaginal discs of *Drosophila melanogaster*. J Neurosci 5:2453–2465.

Jonas P, Phannavong B, Schhuster R, Schröder C, Gundelfinger ED. 1994. Expression of the ligand-binding nicotinic acetylcholine receptor subunit Da2 in the *Drosophila* central nervous system. J Neurobiol 25:1494–1508.

Kankel DR, Ferrus A, Garen SH, Harte PJ, Lewis PE. 1980. The structure and development of the nervous system. In: Ashburner M, Wright TRF, editors. The genetics and biology of *Drosophila*, vol. 2. New York: Academic Press; p 295–368.

Kitamoto T, Salvaterra PM. 1993. Developmental regulatory elements in the 5' flanking DNA of the *Drosophila* choline acetyltransferase gene. Roux's Arch Dev Biol 202:159–169.

Kitamoto T, Ikeda K, Salvaterra PM. 1992. Analysis of cis-regulatory elements in the 5' flanking region of the *Drosophila melanogaster* choline acetyltransferase gene. J Neurosci 12:1628–1639.

Kitamoto T, Ikeda K, Salvaterra PM. 1995. Regulation of choline acetyltrasferase/*lacZ* fusion gene expression in putative cholinergic neurons of *Drosophila melanogaster*. J Neurobiol 28:70–81.

Kitamoto T, Wang W, Salvaterra PM. 1998. Structure and organization of the *Drosophila* cholinergic locus. J Biol Chem 273:2706–2713.

Lutz EM, Tyrer NM. 1988. Immunohistochemical localization of serotonin and choline acetyltransferase in sensory neurones of the locust. J Comp Neurol 267:335–342.

Meinertzhagen IA, O'Neil SD. 1991. Synaptic organization of columnar elements in the lamina of the wild type in *Drosophila melanogaster*. J Comp Neurol 305:232–263.

Merritt DJ, Murphey RK. 1992. Projections of leg proprioceptors within the CNS of the fly *Phormia* in relation to the generalized insect ganglion. J Comp Neurol 322:16–34.

Meyer EP, Matute C, Streit P, Nässel DR. 1986. Insect optic lobe neurons identifiable with monoclonal antibodies to GABA. Histochemistry 84:207–216.

Miller A 1950. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In: Demerec M, editor. Biology of *Drosophila*. New York: Hafner Publishing; p420–534.

Mindek G. 1968. Proliferations- und Transdeterminationsleistungen der weiblichen Genital- Imaginalscheiben von *Drosophila melanogaster* nach Kultur in vivo. Roux's Arch Dev Biol 161:249–280.

Murphey RK, Possidente D, Pollack G, Merritt DJ. 1989. Modality-specific axonal projections in the CNS of the flies *Phormia* and *Drosophila*. J Comp Neurol 290:185–200.

Nässel DR. 1991. Neurotransmitters and neuromodulators in the insect visual system. Prog Neurobiol 37:179–254.

Nässel DR. 1993. Neuropeptides in the brain: a review. Cell Tissue Res 273:1–29.

Nässel DR, O'Shea M. 1987. Proctolin-like immunoreactive neurons in the blowfly central nervous system. J Comp Neurol 265:437–454.

Nayak SV, Singh RN. 1983. Sensilla on the tarsal segments and mouthparts of adult *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). Int J Insect Morphol Embryol 12:273–291

O'Brien MA, Schneider LE, Taghert PH. 1991. In situ hybridization analysis of the FMRFamide neuropeptide gene in *Drosophila*. II.

Constancy in the cellular pattern of expression during metamorphosis. J Comp Neurol 304:623–638.
Palka J, Lawrence PA, Hart HS. 1979. Neural projection patterns from homeotic tissue of *Drosophila* studied in *bithorax* mutants and mosaics. Dev Biol 69:549–575.
Pollack I, Hofbauer A. 1991. Histamine-like immunoreactivity in the visual system and brain of *Drosophila melanogaster*. Cell Tissue Res 266:391–398.
Power ME. 1948. The thoracico-abdominal nervous system of an adult insect D*rosophila melanogaster*. J Comp Neurol 88:347–409.
Restifo LL, White K. 1990. Molecular and genetic approaches to neruotransmitter and neruomodulator systems in *Drosophila*. Adv Insect Physiol 22:115–219.
Salvaterra PM, Vaughn JE. 1989. Regulation of choline acetyltransferase. Int Rev Neurobiol 31:81–143.
Schmidt M. 1997. Distribution of presumptive chemosensory afferents with FMRFamide- or substance P-like immunoreactivity in decapod crustaceans. Brain Res 746:71–84.
Schmidt M, Ache BW. 1994. FMRFamide-like immunoreactivity in presumptive chemosensory afferents of the spiny lobster P*anulirus argus*. Brain Res 315–324.
Schuster R, Phannavong B, Schröder C, Gundelfinger ED. 1993. Immunohistochemical localization of a ligand-binding and a structureal subunit of nicotinic acetylcholine receptors in the central nervous system of *Drosophila melanogaster*. J Comp Neurol 335: 149–162.
Shanbhag SR, Singh K, Singh RN. 1992. Ultrastructure of the femoral chordotonal organs and their novel synaptic organization in the legs of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae). Int J Insect Morphol Embryol 21:311–322.
Singh RN, Nayak S. 1985. Fine structure and primary sensory projections of sensilla on the maxillary palp of *Drosphila melanogaster* Meigen (Diptera: *Drosophila*idae). Int J Insect Morphol Embryol 14:291–306.
Smith SA, Shepherd D. 1996. Central afferent projections of proprioceptive sensory neurons in *Drosophila* revealed with the enhancer-trap technique. J Comp Neurol 364:311–323.
Steller H, Fischbach K-F, Rubin GM. 1987. *disconnected*: a locus required for neuronal pathway formation in the visual system of *Drosophila*. Cell 50:1139–1153.
Stengl M, Homberg U, Hildebrand JG. 1990. Acetylcholinesterase activity in antennal receptor neurons of the sphinx moth *Manduca sexta*. Cell Tissue Res 262:245–252.
Stocker RF. 1994. The organization of the chemosensory system in *Drosophila melanogaster*: a review. Cell Tissue Res 275:3–26.
Stocker RF, Schorderet M. 1981. Cobalt filling of sensory projections from internal and external mouthparts in *Drosophila*. Cell Tissue Res 216:513–523.
Stocker RF, Singh RN, Schorderet M, Siddiqi O. 1983. Projection patterns of different types of antennal sensilla in the antennal glomeruli of *Drosophila melanogaster*. Cell Tissue Res 232:237–248.
Stocker RF, Lienhard MC, Borst A, Fischbach K-F. 1990. Neuronal architecture of the antennal lobe in *Drosophila melanogaster*. Cell Tissue Res 262:9–34.
Sugihara H, Andrisani V, Salvaterra PM. 1990. *Drosophila* choline actyltransferase uses a non-AUG initiation codon and full length RNA is inefficiently translated. J. Biol. Chem, 265:21714–21719.
Takagawa K, Salvaterra PM. 1996. Analysis of choline acetyltransferase protein in temperature sensitive mutant flies using newly generated monoclonal antibody. Neurosci Res 24:237–243.
Taylor B. 1989a. Sexually dimorphic neurons in the terminalia of *Drosophila melanogaster*: I. Development of sensory neruons in the genital disc during metamorphosis. J Neurogenet 5:173–192.
Taylor B. 1989b. Sexually dimorphic neurons of the terminalia of *Drosophila melanogaster*: II. Sex-specific axonal arborizations in the central nervous system. J Neurogenet 5:193–213.
Tix S, Minden JS, Technau GM. 1989. Pre-existing neuronal pathways in the developing optic lobes of *Drosophila*. Development 105:739–746.
Trimmer BA. 1995. Current excitement from insect muscarinic receptors. Trends Neurosci 18:104–111.
White K, Hurteau T, Punsal P. 1986. Neuropeptide-FMRFamide-like immunoreactivity in *Drosophila*: development and distribution. J Comp Neurol 247:430–438.
Wilczek M. 1967. The distribution and neuroanatomy of the labellar sense organs of the blowfly *Phormia regina* Meigen. J Morphol 122:175–201.
Yasuyama K, Kitamoto T, Salvaterra PM. 1995a. Immunocytochemical study of choline acetyltransferase in *Drosophila melanogaster*: an analysis of *cis*-regulatory regions controlling expression in the brain of cDNA-transformed flies. J Comp Neurol 361:25–37.
Yasuyama K, Kitamoto T, Salvaterra PM. 1995b. Localization of choline acetyltransferase-expressing neurons in the larval visual system of *Drosophila melanogaster*. Cell Tissue Res 282:193–202.
Yasuyama K, Kitamoto T, Salvaterra PM. 1996a. Differential regulation of choline acetyltransferase expression in adult *Drosophila melanogaster* Brain J Neurobiol 30:205–218.
Yasuyama K, Kitamoto T, Salvaterra PM. 1996b. Distribution and central projections of putative cholinergic leg sensory neurons in *Drosophila*. Zool Sci 13(Suppl):120.