Differential Regulation of Choline Acetyltransferase Expression in Adult *Drosophila melanogaster* Brain

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SUMMARY

Choline acetyltransferase (ChAT, E.C.2.3.1.6) catalyzes the synthesis of acetylcholine, and is considered to be a phenotypic marker specific for cholinergic neurons. *In situ* hybridization using a nonradioactive cRNA probe identified a large number of cell bodies expressing ChAT mRNA in the cortices of wild-type *Drosophila melanogaster* brain. Strong labeling is remarkable in the cortical regions associated with the lamina and antennal lobe, and also in the median neurosecretory (MNS) cells within pars intercerebralis, suggesting that some of the lamina monopolar neurons, antennal interneurons, and MNS cells are cholinergic. In two temperature-sensitive mutant alleles, Cha^{ts1} and Cha^{ts2}, most hybridization signal disappears after exposure to a restrictive temperature (30°C). Loss of signal is especially evident in the optic lobes. Some centrally located neurons, however, continue to express ChAT mRNA and are thus likely to have expression controlled in a different way than the majority of cholinergic neurons. Immunocytochemistry, using a ChAT specific monoclonal antibody, identified two sets of paired neurons located in the posterior cortex of the brain. These neurons persist in ChAT immunoreactivity even in the Cha^{ts} mutants exposed to restrictive temperature. ChAT mRNA is also detectable in the corresponding cell bodies when Cha^{ts} mutants are held at restrictive temperature. Our findings demonstrate some specific cholinergic neurons in *Drosophila* brain, and indicate that ChAT expression is differentially regulated in particular sets of cholinergic neurons.

Keywords: **cholinergic neurons, *in situ* hybridization, immunocytochemistry, gene expression, temperature-sensitive mutants.**

INTRODUCTION

The nervous system of an organism is composed of neurons with a variety of specific neurotransmitter phenotypes. We are interested in investigating the molecular logic used for the specification and maintenance of chemical phenotypes because these processes are key components of normal synaptic transmission. Choline acetyltransferase (ChAT, E.C.2.3.1.6) is the biosynthetic enzyme responsible for acetylcholine production and is a phenotypically specific component of all cholinergic neurons (e.g., Salvaterra and Vaughn, 1989). Using *Drosophila* as a genetic model system we have studied the DNA regulatory motifs controlling expression of the ChAT gene. Deletion mapping of the 5′ flanking sequence of the *Drosophila* ChAT gene indicated that the 7.4 kb of 5′ flanking DNA contains separable regulatory elements that function in discrete subsets of cholinergic neurons (Kitamoto et al., 1992; Kitamoto and Salvaterra, 1993; Yasuyama et al., 1995).

Previous immunocytochemical studies using monoclonal antibodies for *Drosophila* ChAT have shown wide distribution of ChAT protein in specific regions of the neuropil of adult brain (Buchner et al., 1986; Gorczyca and Hall, 1987; Ikeda and Salvaterra, 1989), but have detected only few, if any, cell bodies that show weak immunoreactivity among cortical cells in the first optic

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ganglia and in the cell layers adjacent to the antennal lobe (Gorczyca and Hall, 1987). *In situ* hybridization was used in a previous study to determine the location of cell bodies expressing ChAT mRNA in *Drosophila* brain (Barber et al., 1989). It was not possible to provide single-cell resolution, however, because of the small size of *Drosophila* cortical interneurons and the relatively high energy of the ^{35}S-labeled probe (Barber et al., 1989). The precise location and morphological organization of ChAT expressing neurons in the central nervous system of *Drosophila* has thus remained undetermined.

One aim of the present study was to localize identifiable ChAT expressing neurons in the *Drosophila* brain that may prove useful for further molecular and physiological analysis of cholinergic neurotransmission. We examined the distribution of ChAT expressing neurons in adult brain by immunocytochemistry using a monoclonal antibody against *Drosophila* ChAT, and by *in situ* hybridization using a nonradioactive cRNA probe specific for *Drosophila* ChAT mRNA. We also utilized P-element transformed flies, carrying 7.4 kb of 5′ flanking DNA from the ChAT gene, which was fused to a *lacZ* reporter gene. These *lacZ* transformants display a β-galactosidase expression pattern that is very similar to that of endogenous ChAT protein (Kitamoto et al., 1992). These three different, but complementary techniques allowed identification of several functionally different cell groups and indicated the involvement of acetylcholine as a neurotransmitter in the cerebral interneurons of wild-type *Drosophila* brain.

We also tested immunoreactivity and *in situ* hybridization in animals carrying a temperature sensitive ChAT mutant allele: Cha^{ts1} or Cha^{ts2} (Greenspan, 1980). Both are thought to be simple point mutations in the structural gene for ChAT (Tajima and Salvaterra, 1990). Previous immunocytochemical and molecular biological studies demonstrated a reduction in ChAT protein levels in the optic lobe (Gorczyca and Hall, 1987; Ikeda and Salvaterra, 1989) as well as mRNA levels (Tajima and Salvaterra, 1992) when animals were exposed to a restrictive temperature. In the present study, we examined the effect of restrictive temperature on the *in vivo* expression of ChAT mRNA. We also extended the immunohistochemical observations to the central brain of Cha^{ts}. Both techniques indicate that ChAT expression is differentially regulated in particular cholinergic neurons in response to a temperature shift.

MATERIALS AND METHODS

Animals

Wild-type (Canton-S) *Drosophila melanogaster*, conditional mutants (Cha^{ts1} and Cha^{ts2}), and P-element transformed flies were reared by standard methods at 18°C. Cha^{ts1} and Cha^{ts2} mutants were isolated and described by Greenspan (1980) and were obtained from Dr. J. Hall. For exposure of Cha^{ts} to restrictive temperature (30°C), 4-day-old female or male flies were put into clean vials (20 flies/vial) containing a diet of cornmeal, and held in an incubator for the desired period of time. Transformants were generated by Kitamoto et al. (1992) and carry a fusion construct consisting of 7.4 kb of 5′ flanking DNA from the ChAT gene driving expression of *Escherichia coli lacZ* gene.

In Situ Hybridization

Preparation of cRNA Probes. The 2.4-kb *Drosophila* ChAT cDNA, containing the entire protein coding sequence (Itoh et al., 1986), was subcloned into pGEM-7Zf(+) plasmid (Promega). Sense and antisense cRNA probes were prepared by transcribing pGEM-7Zf(+) with SP6 or T7 RNA polymerase after linearization with EcoRI or BamHI, respectively. Transcription reactions for digoxigenin labeling were done using the manufacturer's recommended protocol (Boehringer–Mannheim Inc.) and probes were reduced to fragments of approximately 150 bases by limited alkaline hydrolysis. The probes were synthesized, precipitated with ethanol at −80°C for 30 min, and stored at −80°C until use.

Hybridization and Staining. Heads were removed from CO_2 anesthetized animals, frozen in Tissue-TEK O.C.T. compound (Miles) compound, cut into 20-μm thick cryostat sections, and mounted on 3-aminopropyltriethoxysilane-coated slides (Maddox and Jakins, 1987). After air drying the sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature, and then rinsed in PBS. The sections were treated with proteinase K (5 μg/mL) in TE (10 m*M* Tris-HCl pH 7.6, 1 m*M* EDTA) for 10 min at 37°C, rinsed in PBS containing 2 mg/mL glycine for 1 min, then postfixed with 4% paraformaldehyde for 20 min. After washing in PBS, sections were dehydrated through a graded series of alcohol and air dried.

Sections were prehybridized for 2 h at 50°C and hybridized overnight with digoxigenin-labeled probe (0.5 μg/mL) at 50°C. Hybridization was carried out under parafilm in a humid chamber. Hybridization solution consisted of 50% formamide, 5× standard saline citrate (SSC; 1× SSC is 150 m*M* NaCl, 15 m*M* sodium citrate), 2 m*M* EDTA, 2× Denhardt's solution, heat-denatured salmon sperm DNA (500 μg/mL), and yeast transfer RNA (500 μg/mL). After hybridization, the slides were rinsed according to the procedure of Mori et al. (1993)

with some modifications. After washing in 5× SSC for 15 min and in 2× SSC containing 0.1% β-mercaptoethanol for 15 min, the sections were treated with RNase A (50 μg/mL in TE containing 0.5 *M* NaCl) for 30 min at 37°C. The sections were then rinsed in 0.5× SSC, 60% formamide at 55°C for 40 min, 0.1× SSC, 70% formamide at 60°C for 40 min, and 0.1× SSC, 1% β-mercaptoethanol at room temperature for 20 min. Subsequently, the sections were washed in 0.1 *M* Tris-HCl (pH 7.5) containing 0.15 *M* NaCl, and then treated with blocking solution (1% BSA, 0.1% Triton X-100 in the above buffer) at room temperature for 30 min. The sections were incubated with antidigoxigenin-alkaline phosphatase labeled antibody (1:500 to 4000 dilution in blocking solution) for 3 h at room temperature. Staining was detected using a mixture of nitroblue tetrazolium and X-phosphate. Stained sections were mounted with a mixture of glycerol and TE.

For whole-mount *in situ* hybridization, the brains were isolated from the head capsule and fixed and hybridized as described above. The specimens were washed according to Tauz and Pfeifle (1989) and then treated with RNase A solution. After incubation with 1% blocking solution (supplied with the Boehringer kit) for 1 h at room temperature, the specimens were incubated with diluted antidigoxigenin-alkaline phosphatase labeled antibody for 3 h at room temperature.

Immunocytochemistry

Heads were cut from anesthetized flies and fixed in Bouin's fixative (saturated picric acid, 15 mL; formalin, 5 mL; glacial acetic acid, 1 mL) for 15 h at 4°C. Deparaffinized serial sections (12 μm) were incubated with 0.3% H_2O_2 in methanol for 30 min, rinsed in PBS, and then incubated in blocking solution (normal horse serum diluted 1:50 with PBS containing 0.1% Triton-X 100) for 30 min at room temperature. Subsequently, the sections were incubated in diluted monoclonal anti-*Drosophila* ChAT antibody (4B1) produced by Takagawa (in preparation) (0.6 μg IgG/mL in blocking solution) for 20 h at 4°C and rinsed in PBS for 30 min. The Vectastain avidin-biotin-peroxidase complex (ABC) method (Vector Lab) was used for visualization. Sections were incubated in secondary antibody solution (1:200 dilution of biotinylated horse antimouse IgG) for 40 min at room temperature. After washing with PBS, the sections were incubated in ABC solution (1:100) for 40 min at room temperature and rinsed in PBS for 30 min. Color was developed by incubation with a solution of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.01% H_2O_2 in 0.05 *M* Tris-HCl buffer (pH 7.2) for 2–3 min. Stained sections were washed, dehydrated, and mounted with Permount (Fisher Scientific Co.).

For whole-mount preparations, the brains were removed from the head capsule before fixation. The isolated brains were incubated in blocking solution for 2 h at room temperature and incubated in diluted 4B1 for 3 days at 4°C. After overnight washing with PBS, the specimens were incubated in the biotinylated secondary antibody solution (1:200) for 15 h at 4°C and washed overnight with PBS. Subsequently, the specimens were incubated in the ABC solution (1:100) for 15 h at 4°C and washed overnight with PBS. After preincubation with a solution of 0.05% DAB for 1 h in the dark, the specimens were incubated for 5–10 min with a fresh 0.05% DAB solution containing 0.01% H_2O_2. Stained specimens were dehydrated and mounted with Permount.

X-Gal Staining of Transformants

The expression of β-galactosidase from the reporter gene in P-element transformants was detected by staining with X-gal. The brains were removed from the head capsule and fixed with 0.5% glutaraldehyde in PBS containing 0.1% Triton-X 100 (PBS/Triton) for 30 min at room temperature followed by washing in PBS/Triton and 0.1 *M* Tris-HCl buffer (pH 7.4) for 30 min each. Specimens were incubated overnight in the dark at room temperature in X-gal staining buffer containing 10 m*M* Tris-HCl (pH 7.4), 5 m*M* potassium ferrocyanide, 5 m*M* potassium ferrocyanide, 150 m*M* NaCl, 2 m*M* $MgCl_2$, and 0.2% X-gal. After washing in Tris-HCl buffer for 30 min, they were dehydrated, cleared, and mounted with Permount.

RESULTS

Localization of ChAT Expressing Neurons in Wild-Type Brain

In situ hybridization revealed neurons expressing ChAT mRNA in almost the entire cortical region associated with the cephalic and subesophageal ganglion in wild-type flies (Fig. 1). The cytoplasm of stained cell bodies appeared granular and the nucleus was not stained [Fig. 1(B)]. Neuropil regions displayed no staining. A sense control probe did not hybridize to any region of the cortices.

In the optic lobe the lamina cell body region was strongly stained. Two cell body layers were recognized in this region, and the distal layer appeared to be stained darker [Fig. 1(A)]. In the cell body region between the lamina and the medulla, the stained cell bodies were seen on both the anterior and posterior sides of the first chiasm [Fig. 1(A)]. The cells located anterior to the medulla [Fig. 1(A), thick arrow] and posterior to the lobula complex [Fig. 1(A), arrowhead] also exhibited staining. In the subesophageal ganglion, positive neurons were found in the lateral cortical region [Fig. 1(A), double arrowheads] and in the anteroventral cortex situated posterior to the sucking pump [Fig. 1(A), thin arrow]. The cell bodies found in the anterior region of the subesophageal

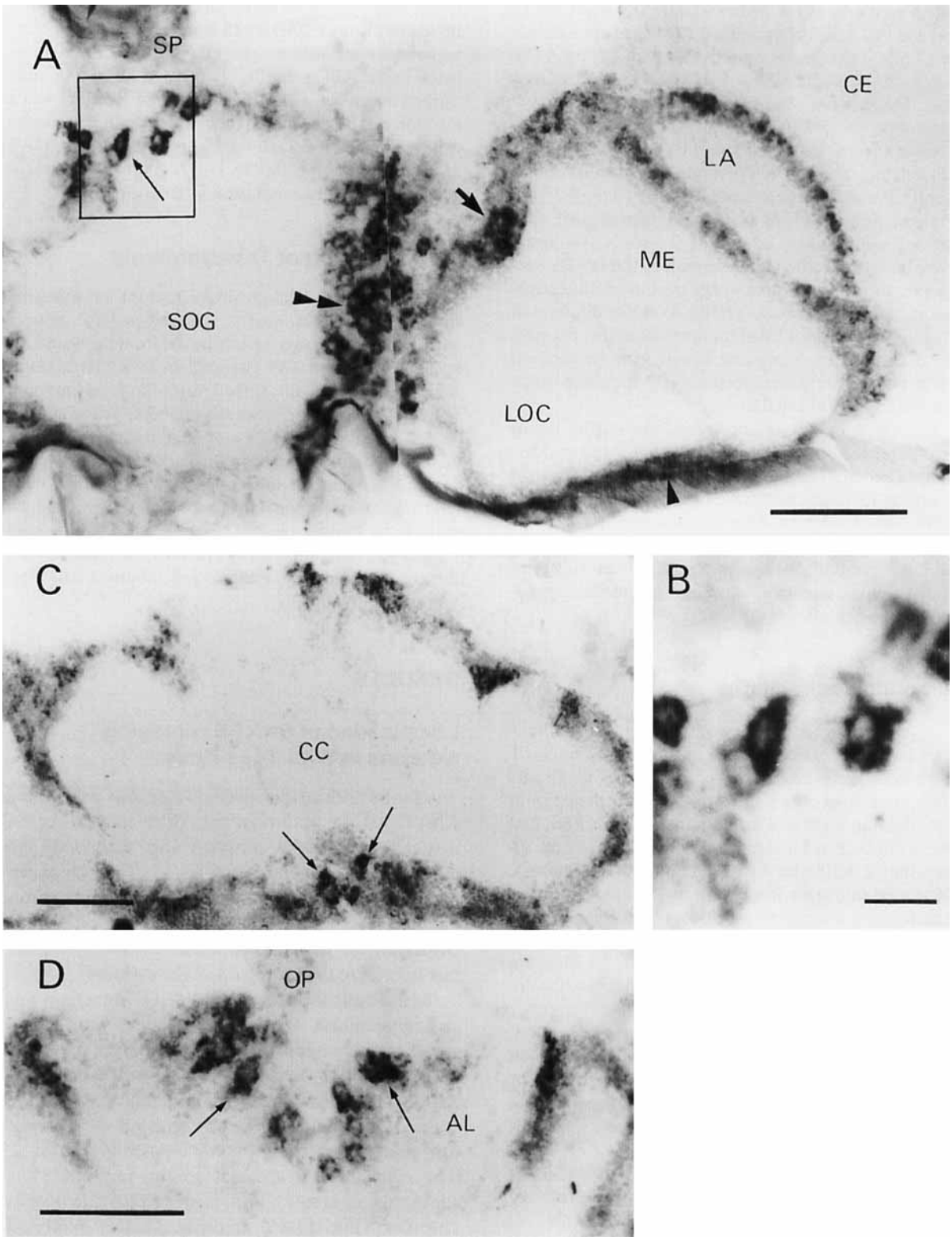


Figure 1 *In situ* localization of ChAT gene transcripts using a digoxigenin-labeled cRNA probes in wild-type brain. (A) A horizontal section showing optic lobe and subesophageal ganglion (SOG). Detection of ChAT mRNA is restricted to the cell bodies in the cortical rind of the brain including the optic lobe. The weak staining in the cortical rind corresponds to

ganglion are larger than those in the lateral cortex [Fig. 1(A)].

In the cephalic ganglion, the cell bodies located in the cortices anterior to or between the right and left antennal lobes, as well as the cortical regions lateral to the antennal lobe expressed substantial amounts of ChAT transcript [Fig. 1(C,D)]. The medially located cell bodies appear larger than the cells located laterally to the antennal lobes [Fig. 1(D)]. The large cell bodies expressing ChAT mRNA appear to be antennal interneurons. Previous immunocytochemical studies using monoclonal antibodies specific for *Drosophila* ChAT demonstrated that the antennal glomerular tract, which carries the axons of the antennal interneurons (Stocker et al., 1990), shows strong ChAT immunoreactivity (Buchner et al., 1986; Gorczyca and Hall, 1987). The ChAT mRNA expressing neurons close to the antennal lobe are probably the source of immunoreactivity in the antennal glomerular tract. In *lacZ* transformants carrying 7.4 kb of 5′ flanking DNA from the ChAT gene, the cell bodies located anterior or lateral to the antennal lobe also show strong X-gal staining as well as the antennal lobe and antennal mechanosensory projection [Fig. 2(A,B)]. Our observations are consistent with the suggestion that some of the antennal interneurons are cholinergic. Large diameter positive cell bodies were also observed in the median region of the posterior rind of the cephalic ganglion at the level of the central complex [Fig. 1(C)].

In the cephalic ganglion, the cRNA probe labels a cluster of cell bodies in the median region of the dorsal rind [Fig. 3(D)]. These cells seem to be identical to the median neurosecretory (MNS) cells within the pars intercerebralis (e.g., Strausfeld, 1976). ChAT immunoreactivity was also detected in the MNS cells. The MNS cells in the adult and late pupal brain showed moderate immunoreactivity [Fig. 3(C,D)]; in the early pupal and late larval brain, the cells showed strong ChAT immu-

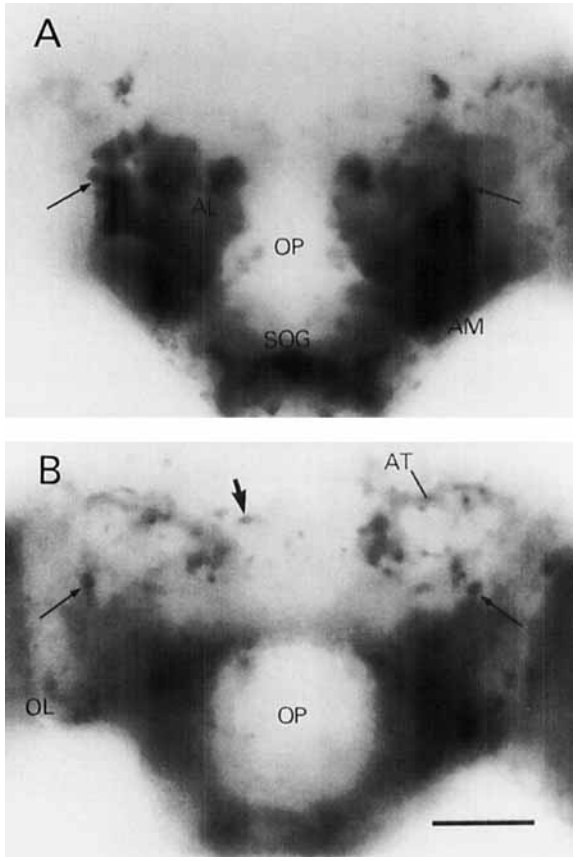


Figure 2 Detection of reporter gene expression in the adult brain of a 7.4-kb *lacZ* transformant. Expression of the reporter gene (β-galactosidase activity) was detected by X-gal staining. (A,B) Frontal views in anterior and posterior portion of brain, respectively. The cell clusters located laterally to the antenna lobe (AL) are strongly stained [arrows in (A)]. The antennal lobes and the antennal mechanosensory projections (AM) also exhibit strong staining. X-gal staining is detected in the neurons corresponding to the PPL neurons [thin arrows in (B)] and PPM [thick arrows in (B)]. The antennal glomerular tracts (AT) also strongly stained. OL, optic lobe; OP, esophagus; SOG, subesophageal ganglion. Scale bars = 50 μm.

specific staining of small cells. The thick arrow and arrowhead indicate the cells located anterior to the medulla (ME) and posteriorly to the lobula complex (LOC), respectively. The double arrowhead and thin arrow show the cells in the lateral cortical region and in the anteroventral cortex situated posterior to the sucking pump (SP), respectively. No staining is seen in the neuropil regions. (B) A magnified view of the cell bodies in the framed area in (A). The cytoplasm shows granular aspect. The nucleus is not stained. (C) A horizontal section at a level of central complex (CC), corresponding to the sectioned level in Figure 5(C). (D) A horizontal section showing antennal lobe (AL) at a level of esophagus (OP). Strongly labeled cell bodies are seen in the median region of the posterior cortex [arrows in (C)] and in the cortex adjacent to the antennal lobe [arrows in (D)]. CE, compound eye; LA, lamina. Scale bars = 50 μm in (A,C,D) and 10 μm in (B).

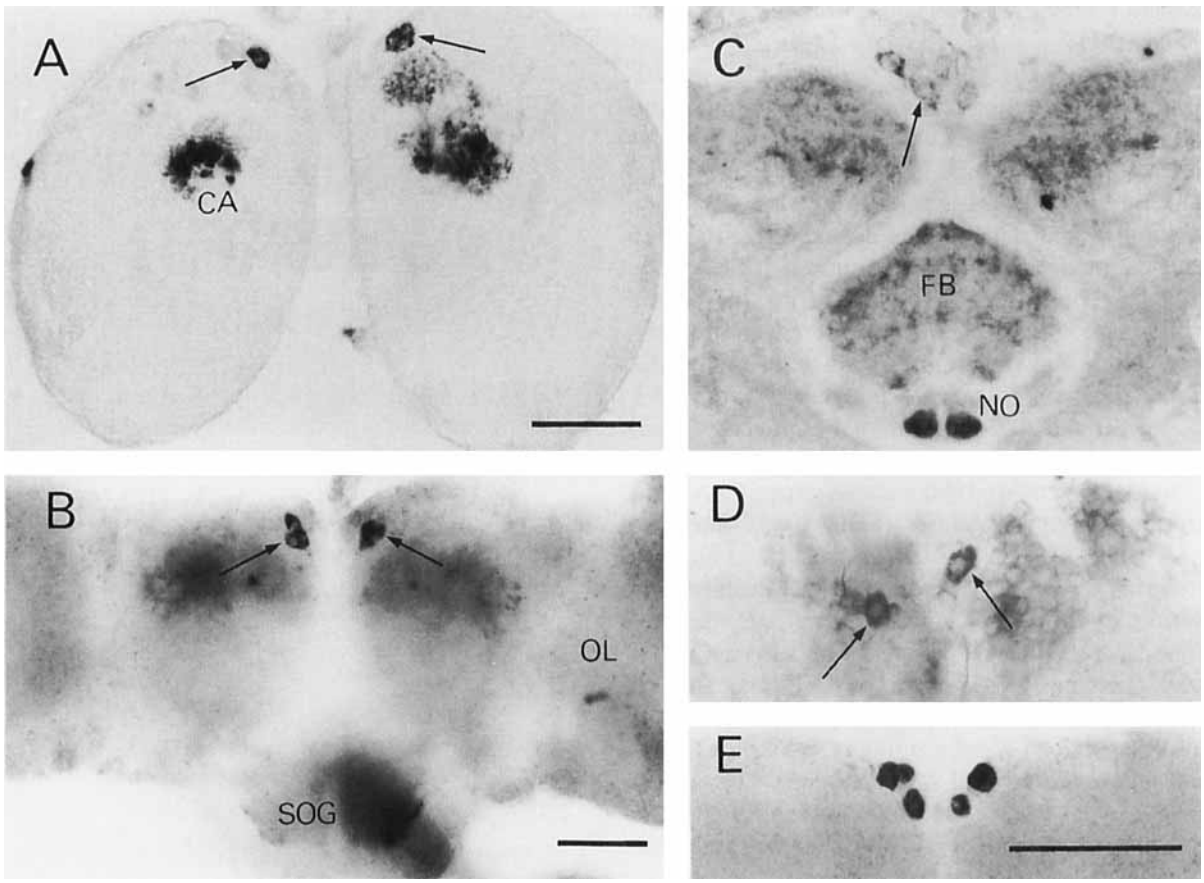


Figure 3 Detection of ChAT expression in the MNS cells within the pars intercerebralis of (A–D) wild-type and (E) *Cha*^{ts} mutant brain. (A) A horizontal section in the dorsal portion of the brain of the late third instar larva. Strong ChAT immunoreactivity is detected in the cell bodies (arrows) that probably correspond to the adult MNS cells. The calyces (CA) are also strongly stained. (B) Frontal view of the whole-mount brain of a pupa from 24 h APF. The MNS cells (arrows) have strong ChAT immunoreactivity. (C) A frontal section of the central part of the brain through the fan-shaped body (FB) of a pupa of 85 h APF. The MNS cells (arrow) and the fan-shaped body indicate moderate ChAT immunoreactivity, while the noduli (NO) are strongly stained. (D) Frontal view of the whole-mount adult brain stained by *in situ* hybridization. ChAT mRNA is detected in the MNS cells (arrows). (E) Frontal view of the whole-mount adult brain from a *Cha*^{ts2} mutant exposed to a high temperature (30°C) for 72 h. The MNS cells exhibit strong ChAT immunoreactivity even after exposure to the high temperature. OL, optic lobe; SOG, subesophageal ganglion. Scale bars = 50 μm.

noreactivity [Fig. 3(A,B)]. Figure 3(C) documents the stained MNS cells in a pupa 85 h after puparium formation (APF). In a 24-h APF pupa, six to eight cells corresponding to the MNS cells are strongly stained [Fig. 3(B)]. In late third instar larvae, the cell clusters with intense immunoreactivity were found on the anterodorsal region in each brain hemisphere. Each cluster consisted of three to four cell bodies [Fig. 3(A)]. These cells are likely to be identical to the MNS cells detected in the adult brain. The MNS cells are also intensively stained in the 7.4-kb *lacZ* transformants (data are not shown). Our observations indicate that some of the neurosecretory cells within the pars intercerebralis of *Drosophila* produce acetylcholine. In the locust, acetylcholine and ChAT immunoreactivity have been reported in the neurosecretory cells of the pars intercerebralis (Geffard et al., 1985).

Effect of Restrictive Temperature on ChAT mRNA Expression in *Cha*^{ts1} and *Cha*^{ts2} Mutants

We examined the expression of ChAT mRNA in the brains of the temperature-sensitive mutant alleles using *in situ* hybridization. In *Cha*^{ts1} mutants

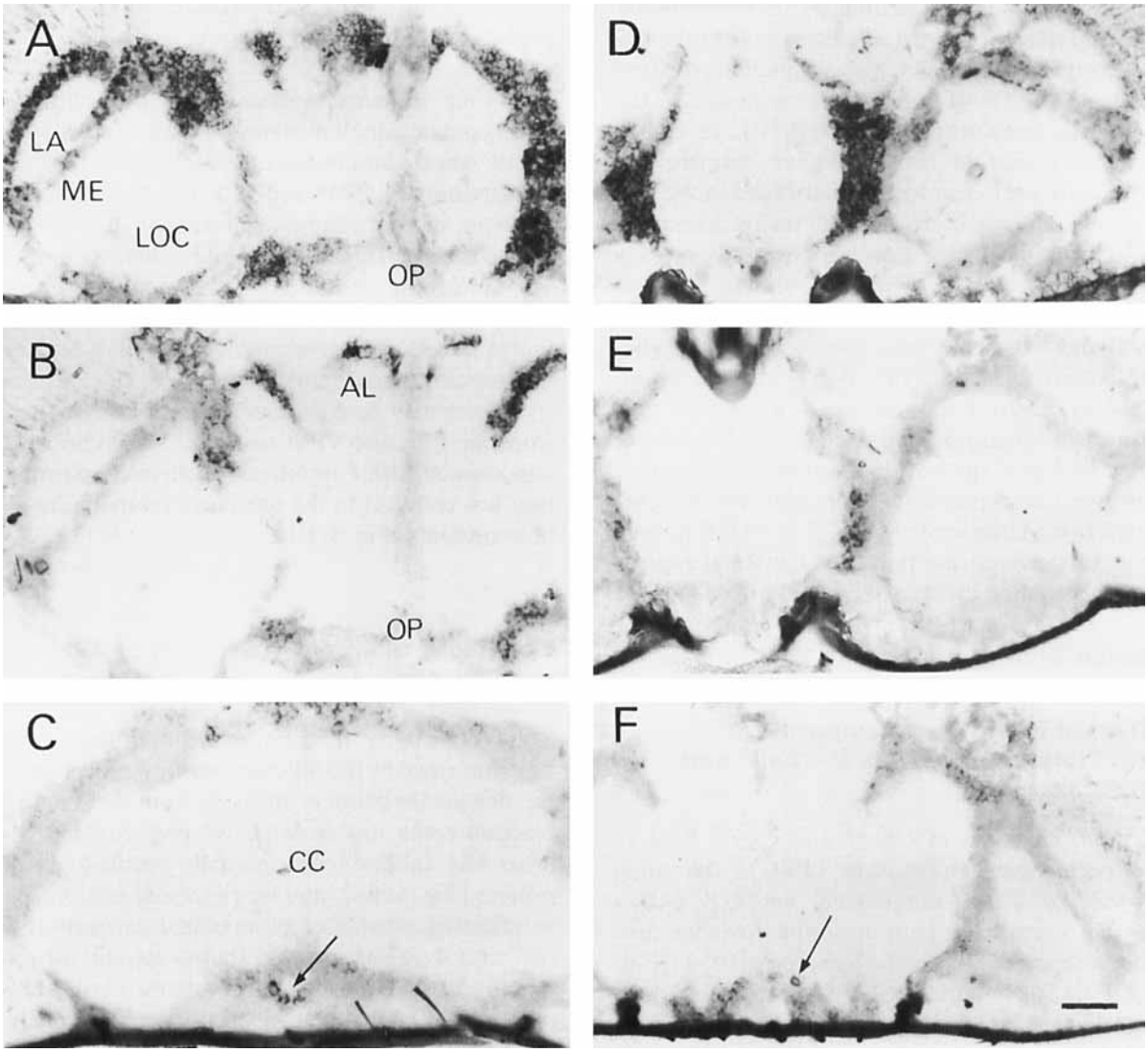


Figure 4 Effect of high temperature of ChAT gene transcript expression in the mutants, [left column; (A,B,C)] Cha^{ts1} and [right column; (D,E,F)] Cha^{ts2}. Horizontal sections. (A,D) The brains of the flies held at 18°C. (B,C,E,F) The brains of the flies after 96-h exposure to a high temperature (30°C). (A,D) At 18°C, ChAT mRNA is substantially detected in the broad regions of the cortical rind of the brain. (B,E) After exposure to the high temperature, the hybridization labels are still observed in the neurons associated with the cerebrum, but not in the neurons of the optic lobe. Note that in both mutants, some cell bodies in the median region of the posterior cortices of the protocerebrum persist in their strong hybridization labeling even after exposure to high temperature (arrows in C,F). (A,B) The sections at the level of the esophagus (OP). (D,E) The sections at the level of subesophageal ganglion (SOG). (C,F) The sections at the level of central complex (CC). AL, antennal lobe; LA, lamina; LOC, lobula complex; ME, medulla. Scale bars = 50 μm.

at a permissive temperature of 18°C, the cRNA probe revealed widespread distribution of hybridization signals in both the cortical regions of the brain and optic lobe [Fig. 4(A)]. Both the pattern and intensity of staining were similar to wild-type flies. After exposing Cha^{ts1} mutants to a restrictive temperature of 30°C for 96 h, however, the positive staining in the optic lobes is almost completely lost [Fig. 4(B)]. Under this condition of restrictive temperature, adult Cha^{ts1} flies are completely paralyzed (Greenspan, 1980). In contrast to the optic lobe, some staining still remained in specific regions of the cerebral cortex [Fig. 4(B)]. At the level of the central complex, the cells located close to the

antennal lobes in the median region of the posterior rind are still stained. Likewise cells in the lateral cortex of the subesophageal ganglion persisted in expressing ChAT mRNA after exposure to the restrictive temperature [Fig. 4(B,C)]. In Cha^{ts2} mutants reared at the permissive temperature, ChAT mRNA is also widely distributed in the cortical region of the brain. The cell bodies associated with the optic lobes, however, are only weakly stained [Fig. 4(D)]. After exposure to the high temperature for 96 h (flies are completely paralyzed), the optic lobe does not show any hybridization reaction [Fig. 4(E)]. In the brain, however, positive staining can still found in specific cortical regions similar to the Cha^{ts1} pattern. The number of the positively stained cell bodies, however, is apparently decreased when compared to mutants kept at 18°C [Fig. 4(E,F)]. Our observations indicate that ChAT mRNA expression is regulated differentially in different parts of the brain of Cha^{ts} mutants in response to a temperature shift.

Effect of Restrictive Temperature on ChAT Immunoreactivity in Cha^{ts1} and Cha^{ts2} Mutants

In the optic lobes of Cha^{ts1} and Cha^{ts2} held at the permissive temperature (18°C), the anti-*Drosophila* ChAT monoclonal antibody (4B1) staining revealed a pattern similar to that previously reported (Ikeda and Salvaterra, 1989) [Fig. 5(A,E)]. The pattern in Cha^{ts1} is similar to that of the wild-type brain (Buchner et al., 1986; Gorczyca and Hall, 1987; Yasuyama et al., 1995): almost all neuropil regions showed ChAT immunoreactivity with the exception of the mushroom body lobes and peduncles [Fig. 5(C)]. Cha^{ts2} at 18°C shows a similar pattern but the intensity of the neuropil staining is significantly lower than Cha^{ts1} [Fig 5(E,G)]. Some structures in Cha^{ts2}, however, are strongly stained. Note the calyces of the mushroom body shown in Figure 5(G). In addition to the broad neuropil staining, two strongly immunoreactive cell body groups are evident in either mutant [Fig. 5(A,C,E,G)]. One of these groups is designated as the posterior paired median (PPM) neurons while the other is termed the posterior paired lateral (PPL) neurons. The PPM neurons are located in the median region of the posterior cortical rind dorsal to the esophagus. They consist of two or three cell bodies. The PPL neurons are composed of two cell bodies located in the posterior cortex laterally to the esophagus. The PPM and PPL neurons as well as the calyx of the mushroom body were also stained in wild-type brain, especially in the brains of newly eclosed flies [Fig. 6(A)].

In Cha^{ts} mutants, most staining in the optic lobe and cephalic ganglion neuropil decreased significantly when animals were held at 30°C for 72 h. The staining of PPM and PPL neurons (Fig. 5), however, did not change as drastically in response to the temperature shift. Weakly stained fibrous structures were also found in the proximal region of the medulla, in the posterior region of the lobula, in the lateral protocerebrum [Fig. 5(E)], and in the subesophageal ganglion [Fig. 5(H)]. These structures may be composed of axons originating from the PPL and PPM neurons. The MNS cells also showed ChAT immunocytochemical staining that was resistant to the restrictive temperature in both mutants [Fig. 3(E)].

Specificity of Immunoreactivity of PPM and PPL Neurons

The specificity of the immunostaining for ChAT was confirmed by the following control procedures: we omitted the primary antibody from the staining reaction (data not shown); we preincubated diluted 4B1 antibody with partially purified ChAT protein [Fig. 6(B)]; and we processed sections by substituting anti-ChAT monoclonal antibody IE6 (0.7 or 1.4 μg/mL of IgG) that is specific for rat ChAT and does not cross-react with the *Drosophila* protein (Crawford et al., 1982), or commercially available (Sigma) mouse myeloma IgG1κ (1.5 μg/mL) instead of the anti-*Drosophila* ChAT (4B1 is an IgG1κ). In each case, no staining was detected in wild-type or the Cha^{ts} mutants exposed to the high temperature for 72 h in PPM, PPL, or MNS neurons. Immunostaining thus depends specifically on the presence of the anti-ChAT monoclonal antibody and is not due to endogenous biotin as observed in other tissues (Ma, 1994) or nonspecific IgG interactions.

In addition to the above negative controls, X-gal staining of the 7.4-kb *lacZ* transformants also indicate that the PPM, PPL, and MNS neurons express ChAT. As shown in Figure 2(B), X-gal staining was detected in the cell bodies corresponding to the PPL and PPM neurons. *In situ* hybridization results using the Cha^{ts} mutants exposed to restrictive temperature also indicate that ChAT mRNA is still detectable in several cell bodies in the posterior rind that have positions similar to PPM and PPL [Fig. 4(C,F)].

DISCUSSION

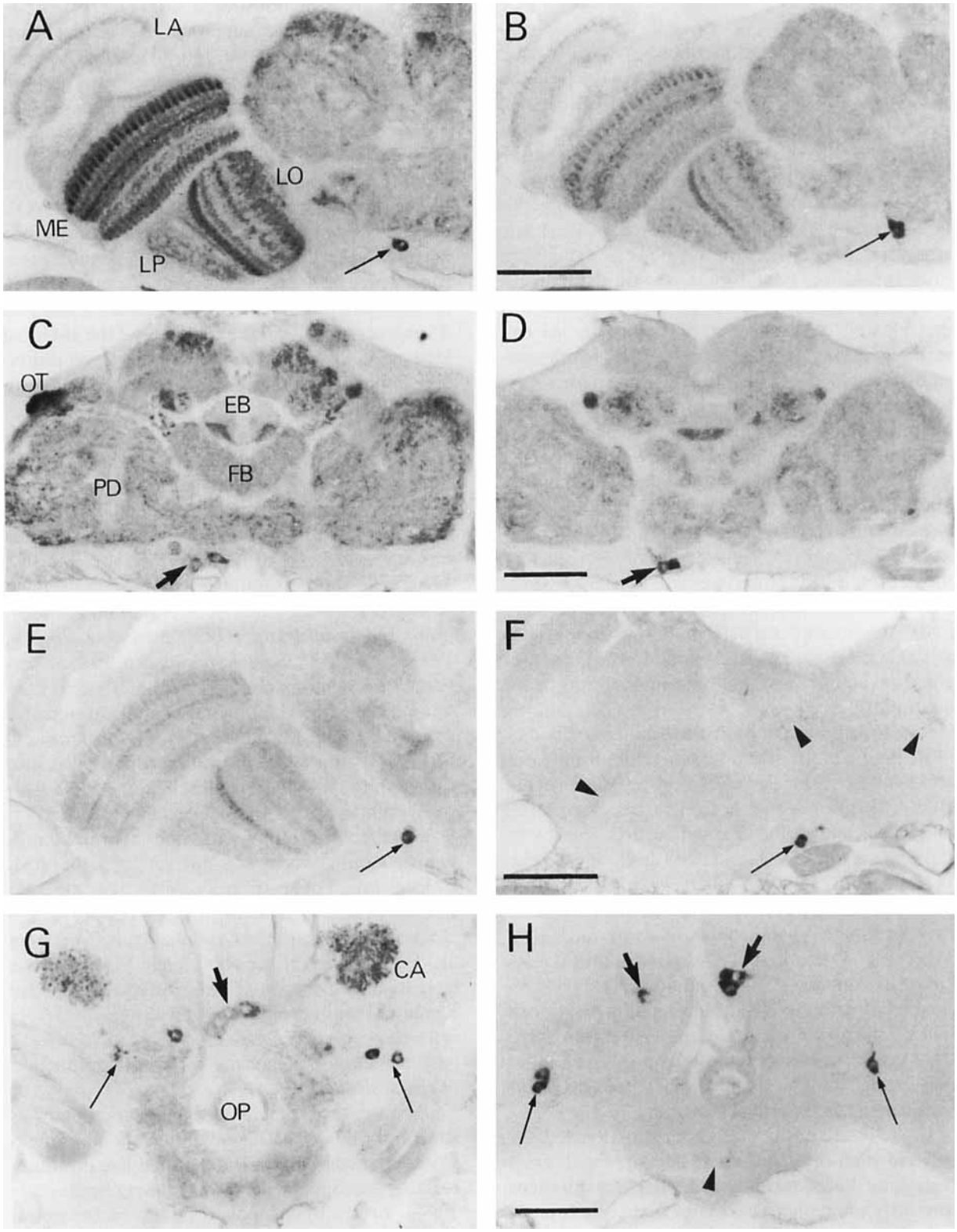
We demonstrated the distribution of the ChAT mRNA expressing neurons in *Drosophila* brain by *in situ* hybridization using a digoxigenin-labeled nonradioactive cRNA probe. The digoxigenin-labeled probe detected ChAT gene transcripts in the cytoplasm of cell bodies with single-cell resolution as contrasted with our previous studies using ^{35}S-labeled probe (Barber et al., 1989). ChAT mRNA was not detected in neuropil regions, and therefore does not seem to be transported to the axon terminals. One limitation of *in situ* hybridization was considerable variability in the intensity of cell staining among different preparations. Semiquantitative results were therefore difficult to obtain by this technique when compared to immunocytochemistry. The variability could be related to the different sizes of *Drosophila* neurons. Larger cell bodies showed better resolution than smaller ones and less variability (see Fig. 1). Variability could also be caused by difficulties in controlling the proteinase K treatment. This step is necessary to remove proteins that interfere with efficient hybridization and different size cells may react differently to this treatment. Unspecific hybridization was not a problem because control probe in the sense orientation did not show any staining in the cortex or neuropil.

The distribution of positively stained cell bodies in wild-type brain agrees in general with previous results using ^{35}S-labeled cRNA probe (Barber et al., 1989). Heavy staining is seen in the lamina cell body layer and in the cerebral cortex. The strong staining seen in the lamina cell body layer [Fig. 1(A)] is consistent with the suggestion based on immunocytochemical observations that L1 and L2 may be cholinergic (Ikeda and Salvaterra, 1989). The cell body cortices of the medulla and the lobula also exhibited positive staining [Fig. 1(A)], in agreement with the X-gal staining of these regions in the 7.4-kb *lacZ* transformants (Kitamoto et al., 1992). Our results and those of previous studies suggest that some of the medulla T neurons or intrinsic neurons are also cholinergic.

In the brain, positive cell bodies are located lateral and anterior to the antennal lobes [Fig. 1(D)], suggesting that some of the antennal lobe interneurons are cholinergic. Two major types of interneurons, local and relay, are distinguished in the antennal lobe of flies (Strausfeld, 1976; Stocker et al., 1990). The relay interneurons connect the antennal lobe with the ipsilateral calyx or lateral protocerebrum by the antennal glomerular tract (Strausfeld, 1976; Stocker et al., 1990). Immunocytochemistry demonstrated strong ChAT immunoreactivity in the antennal glomerular tract (Gorczyca and Hall, 1987) implying that some of the relay interneurons are cholinergic. The positively stained cell bodies close to the antennal lobe are likely to be cholinergic relay interneurons based on their position. Darkly stained cell bodies were also found along the midline on the anteroventral cortex of the subesophageal ganglion [Fig. 1(A)]. On the basis of their position, these neurons may correspond to the giant symmetric relay interneurons, which have extensive symmetric arborization in both antennal lobes (Stocker et al., 1990). In *Manduca,* some local interneurons of the antennal lobe are GABA immunoreactive and exert inhibitory control over the relay interneurons (Boeckh and Tolbert, 1993).

ChAT mRNA and protein expressing neurons were also identifiable within the MNS cell groups associated with the pars intercerebralis [Fig. 3(D)]. A variety of chemical phenotypes were demonstrated for the MNS cells of larger flies by immunocytochemical studies. In the blowfly, MNS cells show immunoreactivity when stained with proctolin, gastrin/cholecystokinin, α-endorphin, and insulin antibodies (Duve and Thorpe, 1979, 1981, 1983; Nässel and O'Shea, 1987). Nässel (1993) demonstrated blowfly MNS cells containing both FMRFamide and small cardioactive peptide B (SCP_B) immunoreactivity. In *Drosophila,* FMRFamide-like immunoreactivity was also shown in the cell bodies (SP3) of some MNS neurons (White et al., 1986; O'Brien et al., 1991); however, colocalization with other neurotransmitters or neuropeptides has not yet been reported. Colocalization of acetylcholine with three different neuropeptides was shown in the accessory radula closer to motoneurons of *Aplysia* (B15; Cropper et al., 1987, 1988). If the cholinergic MNS cells we described in *Drosophila* pars intercerebralis also contain other neuropeptides, these neurosecretory cells may provide a useful model system to investigate molecular mechanisms regulating production of cotransmitters.

Drosophila SP3 larval and adult brain neurons show only moderate FMRFamide staining intensity compared to other FMRFamide-like immunoreactive neurons (White et al., 1986; O'Brien et al., 1991). *In situ* hybridization studies on the developmental regulation of FMRFamide gene expression also showed that hybridization signal in SP3 neurons does not persist beyond the prepupal stage (O'Brien et al., 1991). ChAT immunoreactivity is also easily seen in the MNS cell bodies of larval and early pupal brain and the staining intensity is sig-



nificantly decreased in late pupal and adult brain (Fig. 3). *In situ* hybridization, however, localized ChAT mRNA in the cell bodies of adult MNS cells [Fig. 3(D)]. The developmental regulation of ChAT mRNA expression in larval and pupal MNS cells remains to be examined.

Molecular analysis of ChAT mRNA levels in wild-type and Cha^{ts} mutants shows a ratio of ap-

proximately 1:2:3 for wild-type, Cha^{ts1}, and Cha^{ts2}, respectively, at 18°C (Tajima and Salvaterra, 1992). When animals are held at 30°C for 24 h, both Cha^{ts1} and Cha^{ts2} mRNA levels fall. Our *in situ* hybridization results agree with the molecular analysis for most cholinergic neurons. When it was possible to compare the intensity of hybridization among the genotypes, Cha^{ts1} showed stronger overall labeling at 18°C [Fig. 4(A)] compared to the wild type. Exposure of either mutant to restrictive temperature caused a drastic decrease in the staining intensity of most neurons. This is especially evident in the optic lobes of Cha^{ts1} animals [Fig. 4(B)]. Cha^{ts2} mutants also showed strong labeling in the cortical rind of the cephalic ganglion but rather faint staining in the optic lobes at 18°C [Fig. 4(E)]. This suggest that the overall higher levels of ChAT mRNA seen in Cha^{ts2} (Tajima and Salvaterra, 1992) may be due the strong expression in the cortical rind or perhaps in thoracic ganglion cells because whole flies were analyzed in the biochemical study.

Some centrally located neurons, however, continued to express ChAT mRNA even at the restrictive temperature in the mutants. These neurons were observed in the cortical region close to the antennal lobe [Fig. 4(B)], in the median region of the caudal rind of the protocerebrum [Figs. 4(C,F)], and in the lateral cortex of the subesophageal ganglion [Fig. 4(E)]. These neurons obviously do not respond to the restrictive temperature paradigm the same way as most of the cholinergic neurons. It is possible that the large size of these neurons may contribute to their persistent ChAT expression. We do not believe, however, that this is the only explanation because some of the large ChAT mRNA positive neurons in the lateral cortex of the subesophageal ganglion (Fig. 1) lose their staining in Cha^{ts} animals at restrictive temperatures [compare Fig. 4(D) with 4(E)]. Regulation of ChAT expression may thus be different in different subsets of cholinergic neurons. We also stained some of the Cha^{ts} animals with a commercial anti-FMRFamide antibody and noted no significant differences in staining intensity or pattern when the flies were held at the *Cha* restrictive temperature for 72 h (unpublished data). This indicates that the effect of temperature shows some specificity for ChAT expression.

Immunocytochemistry using a new anti-ChAT monoclonal antibody (4B1) revealed a similar immunolabeling pattern in the optic lobes of the Cha^{ts} mutants as previously reported by Ikeda and Salvaterra (1989). In the present study we also identified two sets of neurons (PPM and PPL), which showed strong ChAT immunoreactivity even in Cha^{ts} animals exposed to restrictive temperature (Fig. 5). The specificity of the immunostaining for these neurons was confirmed by several types of control experiments. ChAT mRNA was also detectable in the cortical regions near the positions of the PPL and PPM neurons, after exposing the mutants to restrictive temperature [Figs. 4(C,F)]. This indicates that PPL and PPM neurons continue to express ChAT transcripts even at restrictive temperature. ChAT immunoreactivity of the PPL and PPM neurons even appeared to be stronger in Cha^{ts} mutants held at the high temperature when compared to specimens at the permissive temperature (Fig. 5). These histochemical aspects of the PPL and PPM neurons in Cha^{ts} mutants are reminiscent of our previous biochemical results that showed increasing ChAT activity and mRNA levels after exposure of wild-type animals

Figure 5 Effect of high temperature on the immunoreactivity to ChAT antibody in the right brain of mutants (A–D) Cha^{ts1} and (E–H) Cha^{ts2}. (A,C,E,G) Left column, 18°C; (B,D,F,H) right column, after 72-h exposure to high temperature (30°C). (A,B,E,F) Horizontal sections showing optic lobe. (C,D) Horizontal sections at the level of central complex. (G,H) Frontal sections in the posterior portion of the brain. At 18°C, the antibody reveals widely distributed ChAT immunoreactivity in the neuropil regions of the (A,E) optic lobe and (C) brain, and also in two sets of cell body groups (PPL and PPM neurons, indicated by thin and thick arrows, respectively). Intensity of the staining of the optic lobe in (E) Cha^{ts2} is much weaker when compared to that in (A) Cha^{ts1}. (B,D,F,H) Exposure to high temperature produces apparent reduction of the immunoreactivity in the mutant brains. Although almost all the staining disappears from the neuropil regions in (F,H) Cha^{ts2}, the PPL and PPM neurons still show strong immunoreactivity, as well as in (B,D) Cha^{ts1}. Note that weakly stained fibrous structures are seen in the optic lobe, protocerebrum, and subesophageal ganglion (arrowheads in F,H). CA, calyx; EB, ellipsoid body; FB, fan-shaped body; LA, lamina; LO, lobula; LP, lobula plate; ME, medulla; PD, peduncle; OP, esophagus; OT, optic tubercle. Scale bars = 50 μm.

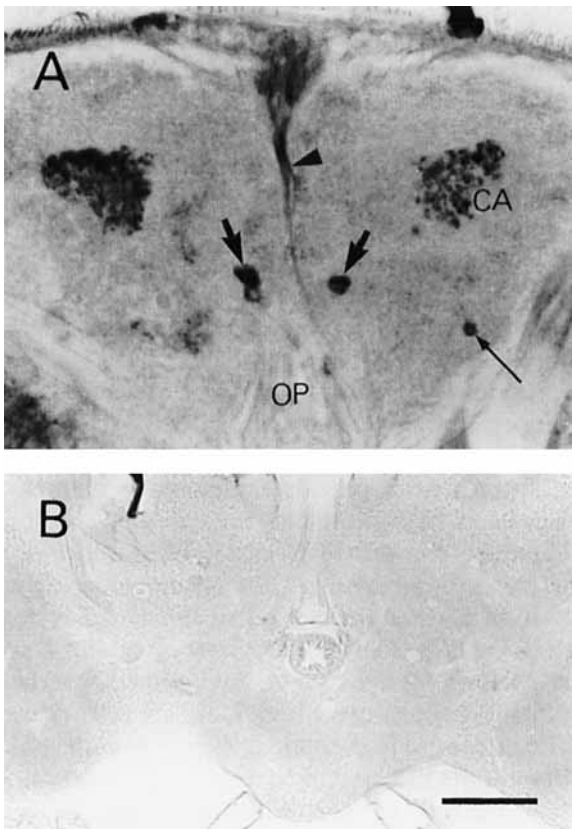


Figure 6 ChAT immunoreactivity in the PPL and PPM neurons of the wild-type brain. Frontal sections in posterior portion of brain. (A) A slightly oblique section from a fly at 1 h after eclosion. The PPL (thin arrow) and PPM (thick arrow) neurons, the calyx (CA), and the axons of the ocellar interneurons (arrowheads) are strongly stained. (B) A section processed by the preabsorbed diluted antibody with the ChAT protein. The staining is completely deleted from all structures. Scale bar = 50 μm.

to the high temperature (Salvaterra and McCaman, 1985; Tajima and Salvaterra, 1992). Thus, the PPL and PPM could be regarded as neurons that exhibit a ChAT expression pattern similar to the wild type, even in the Cha^{ts} brain. Tajima and Salvaterra (1992) proposed that a positive feedback mechanism regulates the level of ChAT mRNA and speculated that a temperature dependent failure of cholinergic synaptic transmission in the Cha^{ts} mutants may be responsible for reduced expression at restrictive temperature. This proposal predicts that the PPL and PPM neurons in the Cha^{ts} mutants maintain synaptic transmission even at a restrictive temperature. If ChAT expression is indeed positively regulated by synaptic transmission, a possible explanation for the persistence of ChAT expression in PPM and PPL may be the use of a cotransmitter. Further studies would be required to investigate this possibility. It should also be mentioned that weakly stained fine fibrous structures were found in the neuropil regions of the medulla and the lateral protocerebrum after Cha^{ts2} mutants' exposure to restrictive temperature [Figs. 5(F,H)]. These fibrous structures could be the axons originating from PPL and PPM somata and may provide an interesting system to investigate the feedback regulation of ChAT expression using electrophysiological and/or fine structural analyses.

Previous (Barber et al., 1989) and present *in situ* hybridization studies demonstrated the existence of a large number of ChAT mRNA expressing neurons in adult *Drosophila* brain. In contrast, immunocytochemistry using anti-ChAT antibodies failed to localize the protein product in most cell sommata in spite of strong and widespread staining of neuropil regions (Buchner et al., 1986; Ikeda and Salvaterra, 1989). A number of possibilities have been proposed to explain the apparent discrepancy in intracellular localization of transcripts and protein including a rapid transport of ChAT protein away from the cell body to axon terminals and/or the masking of the antigenic epitopes in some unknown way prior to ChAT molecules reaching their terminal destination (Barber et al., 1989). No direct information is available to support or refute any possibility, but evidently the PPL and PPM neurons are different than most of the cholinergic neurons in regard to the distribution of ChAT protein.

Our results using *in situ* hybridization demonstrate a large number of *Drosophila* cortical neurons expressing ChAT gene transcripts, and our observations support the notion of differential regulation of ChAT expression for different types of cholinergic neurons in the Cha^{ts} mutants. Furthermore, our immunocytochemical study in Cha^{ts} mutants identifies two sets of cholinergic neurons (PPL and PPM neurons), in which the ChAT expression may be controlled in a different manner from other cholinergic neurons.

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