Replacement of the Glycoinositol Phospholipid Anchor of *Drosophila* Acetylcholinesterase with a Transmembrane Domain Does Not Alter Sorting in Neurons and Epithelia but Results in Behavioral Defects

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Submitted June 28, 1995; Accepted January 24, 1996
Monitoring Editor: Keith Yamamoto

Drosophila has a single glycoinositol phospholipid (GPI)-anchored form of acetylcholinesterase (AChE) encoded by the *Ace* locus. To assess the role that GPI plays in the physiology of AChE, we have replaced the wild-type GPI-AChE with a chimeric transmembrane form (TM-AChE) in the nervous system of the fly. *Ace* null alleles provided a genetic background completely lacking in endogenous GPI-AChE, and *Ace* minigene P transposon constructs were used to express both GPI- and TM-AChE forms in the tissues where AChE is normally expressed. Control experiments with the GPI-AChE minigene demonstrated a threshold between 9 and 12% of normal AChE activity for adult viability. *Ace* mutant flies were rescued by GPI-AChE minigene lines that expressed 12–40% of normal activity and were essentially unchanged from wild-type flies in behavior. TM-AChE minigene lines were able to rescue *Ace* null alleles, although with a slightly higher threshold than that for GPI-AChE. Although rescued flies expressing GPI-AChE at a level of 12% of normal activity were viable, flies expressing 13–16% of normal activity from the TM-AChE transgene died shortly after eclosion. Flies expressing TM-AChE at about 30% of normal levels were essentially unchanged from wild-type flies in gross behavior but had a reduced lifespan secondary to subtle coordination defects. These flies also showed reduced locomotor activity and performed poorly in a grooming assay. However, light level and electron microscopic immunocytochemistry showed no differences in the localization of GPI- and TM-AChE. Furthermore, endogenous and ectopic-induced expression of both AChEs in epithelial tissues of the adult and embryo, respectively, showed that they were sorted identically. Most epithelial cells sorted GPI- and TM-AChE to the apical surface, but cuticle-secreting epithelia sorted both proteins basolaterally. Our data suggest that rather than having a primary role in protein sorting, the GPI anchor of AChE plays some other more subtle cellular role in neuronal physiology.

INTRODUCTION

Cell-surface proteins that are anchored in the plasma membrane by covalent linkage to a glycoinositol phos-

pholipid (GPI)¹ are found in all eukaryotes. Six of seven known GPI-anchored proteins in *Drosophila* are

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¹ Abbreviations used: AChE, acetylcholinesterase; CNS, central nervous system; GPI, glycoinositol phospholipid; HSV gC, herpes simplex virus glycoprotein C; PB, phosphate buffer; PBS, phosphate-buffered saline; PIPLC, phosphatidylinositol-specific phospholipase C; TM, transmembrane

restricted to the nervous system, including acetylcholinesterase (AChE) and the cell adhesion molecules chaoptin, fasciclin I, a minor alternatively spliced form of fasciclin II, and connectin (reviewed by Bunch and Brower, 1993). Since some proteins have both GPI-anchored and peptide-anchored forms, GPI anchors are likely to confer unique functional properties to proteins. The clearest picture of a possible GPI function comes from studies of cultured mammalian cells, where in general a GPI anchor appears to act as an apical, and possibly axonal, targeting signal in polarized epithelial cells and in some neurons, respectively (Lisanti *et al.*, 1989; Dotti *et al.*, 1991; reviewed by Matter and Mellman, 1994), and to cluster in cholesterol-rich plasma membrane invaginations called caveolae (Rothberg *et al.*, 1990). Targeting may result from the segregation of GPIs in membrane microdomains that can include glycosphingolipids and cholesterol (Brown and Rose, 1992) and the cytoplasmic tyrosine kinases *lck*, *fyn*, or *yes* (reviewed in Brown, 1993). This association of GPIs with tyrosine kinases might explain some reports that GPI-anchored proteins can transduce extracellular signals even though they do not span the lipid bilayer. Comparison of T lymphocyte antigens attached either to a GPI or to a transmembrane peptide showed that only the GPI-linked antigens could activate T cells when cross-linked by antibody (reviewed by Robinson, 1991). Elkins *et al.* (1990) demonstrated that, while single mutations in either the *Drosophila abl* tyrosine kinase homologue or GPI-anchored fasciclin I produced only mild defects in the developing fly central nervous system (CNS), double mutant embryos showed severe derangements in the CNS scaffold. Double mutant combinations of *abl* and other cell adhesion molecules with transmembrane peptide anchors expressed in the same cells did not show a similar genetic interaction. However, other studies have failed to detect a necessary signaling role for GPIs. Lee *et al.* (1993) showed that transfected cells expressing either wild-type GPI-anchored CD14 or chimeras anchored by transmembrane domains were able to generate the same biochemical responses to stimulation with lipopolysaccharide ligand in vitro. Barth *et al.* (1994) replaced the wild-type GPI-anchored form of the contact site A glycoprotein of *Dictyostelium* with a chimeric transmembrane form. The chimera showed the same activity as the wild-type contact site A in a cell adhesion assay but had a shorter half-life at the cell surface. Although these studies offer some insights, experiments in cultured cells can provide only limited information on potential GPI functions in vivo because many GPI-anchored proteins are involved in interactions or communication between cells.

To address the role of GPI anchoring in a whole animal with a protein whose function is simple and well understood, we have focused on *Drosophila*

AChE. AChE terminates synaptic transmission at cholinergic synapses by hydrolyzing acetylcholine, the major excitatory neurotransmitter in the insect CNS. The *Ace* locus of *Drosophila* encodes a single form of GPI-anchored AChE (GPI-AChE; Gnagey *et al.*, 1987; Fournier *et al.*, 1989), and *Ace* mutant alleles (Green-span *et al.*, 1980; Hilliker *et al.*, 1981) that express very low AChE activity are recessive lethal (Hall and Kankel, 1976). As with many *Drosophila* genes involved in synaptic transmission (e.g., DiAntonio *et al.*, 1993; Zinsmaier *et al.*, 1994), animals homozygous for lethal *Ace* alleles complete embryogenesis but die trapped in the eggshell as pharate first instar larvae. Although the CNS of these mutants appeared normal at the light microscopic level, an ultrastructural study showed degenerative changes in CNS neurons (Chase and Kankel, 1988). Therefore, lethality probably does not result from the loss of some developmental function of AChE per se, but from the unopposed action of acetylcholine. To gain insight into the roles the GPI anchor may play in the physiology of insect AChE, we have genetically replaced the wild-type GPI-AChE with a chimeric transmembrane form (TM-AChE) and an unanchored, secreted form. The construction, cell culture expression, and biochemical characterization of these forms are described elsewhere (Incardona and Rosenberry, 1996), as is the genetic replacement with the secreted form (Incardona and Rosenberry, unpublished data). Utilizing an *Ace* minigene construct and several combinations of *Ace* lethal alleles, we show that TM-AChE can in general substitute for GPI-AChE. However, animals expressing TM-AChE have behavioral defects despite no apparent difference in cellular localization. Although there is a large amount of literature suggesting that GPIs provide a sorting function in various cultured cells, our study is the first to test this hypothesis in an intact organism.

MATERIALS AND METHODS

Drosophila Stocks

The following *Ace* lethal alleles were examined: null alleles *B21-5*, *H36*, and *H89*, and severe hypomorphs *l26*, *lm15*, and *lm38*. The semi-lethal hypomorphic allele *lm35* and deletion chromosome *Df(3R)l26d* (87D11-13; 87E3-5) were also used. All stocks were maintained and crosses performed at room temperature on standard cornmeal/molasses agar in pint bottles or 8-dram vials. *Ace* alleles were balanced with either *MRS*, *MKRS*, *TM3Sb*, *TM6B*, or *TM3Ser y+*. Balancer chromosomes were described by Lindsley and Zimm (1992).

Subcloning of Wild-type and Mutant Minigene Constructs

Construction of the 5.8-kb *Ace* minigene was described by Hoffmann *et al.* (1992). To generate transformants in a *white* background rather than a *rosy* background, the minigene was obtained as a 5.8-kb *Sall* fragment from *pryAce* (Hoffmann *et al.*, 1992) and subcloned into the *Xho*I site of the P element transformation vector pCaSpeR-4 (Piotto, 1988) to generate *pwAce* (Figure 1). TM-AChE

cDNA was constructed by inserting a sequence corresponding to the transmembrane and cytoplasmic domains of herpes simplex virus-1 glycoprotein C (HSV-1 gC) in place of the C-terminal GPI-anchoring signal in GPI-AChE cDNA (Incardona and Rosenberry, 1996). The segment of the TM-AChE coding sequences containing the altered C-terminus was subcloned into pwAce via the *KspI* and *Asp718* sites (Figure 1) to produce pwTM. To express GPI-AChE and TM-AChE from the *Drosophila hsp70* promoter, the 3'-untranslated region of the *hsp70* gene in the vector pCaSpeR-hs (Pirota, 1988) was removed as a single 500-bp *BamHI* fragment, and the gel-purified vector was recircularized to generate pCaSpeR-hsΔ. The deleted sequence has been shown to destabilize the mRNA during recovery from heat shock (Petersen and Lindquist, 1989). Isolated 3.3-kb *Asp718* fragments containing GPI-AChE and TM-AChE cDNAs (Incardona and Rosenberry, 1996) were blunted by filling the 5' overhangs with the Klenow fragment of DNA polymerase I, ligated to *NotI* linkers, and inserted into the *NotI* site of the pCaSpeR-hsΔ polylinker to generate phsE18 and phsTM, respectively.

Germline Transformation

Vector DNAs containing AChE minigenes and the transposase-encoding helper plasmid phs π (Steller and Pirota, 1986) were purified by two rounds of CsCl gradients or on Qiagen columns and co-injected into embryos by established protocols (Karess, 1986) to generate transformants. Injected embryos were of genotype *y w*¹¹¹⁸. Additional minigene lines were generated by mobilization of a P element insert. Briefly, an X-linked transgene insert with weak *white+* expression was mobilized in males by crossing in the Δ2-3 99B chromosome as a source of constitutively expressed transposase (Robertson *et al.*, 1988). New lines with autosomal inserts were selected unambiguously in sons of such males, and new X-linked lines were selected in the daughters by virtue of higher *white+* expression. The locations of the inserts were not determined beyond the linkage group. All P element minigene inserts used in this study were homozygous viable except for two GPI-AChE inserts G6 and G7, and parent stocks carrying GPI-AChE or TM-AChE transgenes in a wild-type (i.e., *y w*) background showed no obvious phenotypic difference from nontransformed flies.

Extraction and Assay of AChE

AChE assays in homogenates followed the procedure of Ellman *et al.* (1961), as modified by Rosenberry and Scoggin (1984). To determine the percentage of AChE activity in rescued flies relative to wild-type (*y w*) controls, single whole flies were homogenized in 50 μ l DSS buffer (20 mM sodium phosphate, pH 6.9, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 5 mM *N*-ethylmaleimide, and 1.0 μ g/ml each of leupeptin and pepstatin) and centrifuged, and 40 μ l of supernatant was assayed. Control AChE activity of *y w* females was determined to be 7.0 ± 0.7 mU ($N = 23$). Nondenaturing polyacrylamide gel electrophoresis (ND-PAGE) in 0.5% Triton X-100 with histochemical AChE activity staining and digestion with phosphatidylinositol-specific phospholipase C (PIPLC) were conducted as described elsewhere (Incardona and Rosenberry, 1996).

Behavioral Tests

An assay of general locomotor activity was based on that described by Connolly (1966) and Hall *et al.* (1980). Flies were collected from low-density cultures within 24 h of eclosion and stored on fresh food for 24 h further. Locomotor activity was quantitated by observation of single flies under a 9-cm watch glass placed over paper with a 1×1 cm grid. A fly was placed under the watch glass without anesthesia, allowed to adjust for 2 min, then locomotor activity was recorded by counting the number of grid lines crossed in 1 min. Each fly was monitored for five 1-min periods, and the counter was reset for each period. During the same testing periods,

transfers between the paper grid and the watch glass were scored on a separate counter as a measure of climbing ability. A mean locomotor activity (lines crossed/minute) for each individual was calculated from the five determinations. The mean locomotor activity score of each genotype was obtained from 15–20 individuals. All assays were performed in the late afternoon.

The grooming assay was performed according to the method of Phillis *et al.* (1993), with additional details provided by R.K. Murphy (personal communication). Briefly, 20–30 4-day old flies collected from uncrowded cultures were transferred without anesthesia to a plastic vial containing 3 mg of yellow dust. Two yellow dusts were used with similar results: reactive Yellow 86 (Procion yellow, Brooks and Flynn, Petaluma, CA) and folic acid powder (ICN Biochemicals, Costa Mesa, CA). The flies were coated with dust by a standardized series of shakes and taps, then transferred to a series of two more empty vials and shaken to remove excess dust. Finally the coated flies were transferred to a grooming chamber (Phillis *et al.*, 1993) and allowed to groom for 2 h before scoring under anesthesia with a dissecting microscope. When the parental wild-type (i.e., *y w*) stock used for obtaining the transgenic lines was assayed for grooming behavior, it was observed that the last surface to be cleaned was the notum (the dorsal surface of the thorax). Within 2 h, most *y w* flies would clean the head, wings, abdomen, and notum, while 18% ($N = 66$) still had dust remaining on the notum. We therefore chose to define flies as "dusty" if they had dust remaining on the head, wings, or abdomen. With these criteria, no *y w* flies were scored as ungroomed (dusty). All strains of flies were assayed in parallel in a room with uncontrolled but low humidity.

Immunocytochemistry on Cryostat Sections

Heads with or without the thorax attached were dissected from anesthetized flies and transferred to 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7). Proboscises were removed with fine scissors and a flap of cuticle was removed from the occiput with a razor chip. Legs and wings were cut off at the base and holes were cut in the dorsal cuticle to allow better penetration of fixative. The tissue was fixed at room temperature for 2–4 h, transferred to 20% sucrose in phosphate-buffered saline (PBS; pH 7.4) overnight at 4°C, and frozen onto cryostat chucks in OCT (Miles, Indianapolis, IN) by submersion into a liquid nitrogen slush supercooled under vacuum. Sections (10 μ m) were cut on a Bright Instruments 5030 cryostat, recovered on gelatin-coated slides, and allowed to air dry at room temperature. After blocking in PBS with 0.2% Triton X-100 (PBT) containing 0.2% bovine serum albumin (BSA) and 1% normal goat serum, sections were processed for horseradish peroxidase immunocytochemistry as described in Ashburner (1989) with primary affinity-purified *Drosophila* AChE rabbit antibodies (Incardona and Rosenberry, 1996) diluted 1:1000, goat anti-rabbit biotinylated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:1000, and avidin-horseradish peroxidase complex (ABC Elite Kit, Vector Labs, Burlingame, CA) diluted 1:150. Immunoreactivity was visualized by incubation for 5–7 min in Very Intense Purple substrate (VIP, Vector Labs) mixed according to the manufacturer's directions. AChE histochemistry was performed on parallel sections as described by Tago *et al.* (1986). Sections were mounted under a coverslip in glycerol and photographed on a Zeiss Axioplan Universal microscope.

Immunoelectronmicroscopy

Adult heads were dissected as described for cryostat sections, fixed for 3 h in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7 (PB), transferred to fresh PB, and embedded individually in blocks of 5% agar in PB. Sections (50 μ m) were cut on a vibratome bathed in PB and placed into PB in a 48-well plate with a fine paintbrush for all further processing. After overnight incubation at 4°C with affinity-purified anti-AChE antibodies diluted 1:1000 in PBS (7.4) plus 0.4% BSA, sections were shifted to room temperature

for 2 h and all further treatments. Sections were washed three times with PBS, incubated with biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:1000 in PBS plus 0.4% BSA for 2 h, given three washes (PBS), incubated with avidin-horseradish peroxidase complex (ABC-Elite, Vector Labs) diluted 1:100 in PBS for 2 h, again given three washes (PBS), and exposed to a horseradish peroxidase substrate solution consisting of 0.5 mg/ml diaminobenzidine (Polysciences, Warrington, PA), 0.0015% H_2O_2 , 0.03% cobalt chloride, and 0.03% nickel ammonium sulfate in 0.05 M Tris, pH 7.5. The reaction was allowed to proceed for no longer than 10 min before washing with water. The sections were then fixed overnight at 4°C in 3% glutaraldehyde in PB, given three washes in 0.1 M sodium cacodylate, treated with 1% OsO_4 in 0.1 M cacodylate for 2 h at room temperature in the dark, washed three times in water, and dehydrated through a series of ethanol washes at 25%, 50%, 75%, and three times in 100%. The sections were then treated with propylene oxide and infiltrated with LX-112 resin. After embedding on pre-hardened blocks of LX-112, 70-nm thin sections were cut in the same plane as the vibratome sections using a Reichert-Jung Ultracut E ultramicrotome. Immunolabeled thin sections were not counterstained, but some control sections were stained with lead citrate to assess fixation. Thin sections on copper grids were viewed and photographed on a Jeol 100CX transmission electron microscope. Analysis of GPI-AChE and TM-AChE utilized 100 micrographs each for immunoreactivity and 20 micrographs each for counterstaining, and 20 micrographs of wild-type tissue were examined for control immunoreaction with an irrelevant primary antiserum.

Heat Shock Treatment and Immunocytochemistry on Embryos

Embryos collected over a 24-h period from hsE18- and hsTM-transformed flies were subjected to a 1-h heat shock at 37°C, allowed to recover for 5 h, and transferred to small Nitex mesh baskets in the wells of a multiwell plate. After successive washes with water, 0.02% TX-100, and 0.15 M NaCl and treatment with 75% bleach for 15 min to remove the chorions, embryos were washed copiously with water followed by 0.15 M NaCl. The baskets were then placed in heptane and the embryos transferred to glass vials in heptane. An equal volume of fresh 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7) was added for fixation, and the vials were agitated vigorously for 15 min. The aqueous phase was removed and an equal volume of methanol was spritzed onto the embryos remaining in heptane. After shaking for < 1 min, fixed and permeabilized embryos that sank were transferred back to the Nitex baskets in methanol. After 15 min the embryos were washed three times with PBT, blocked in PBT containing 0.4% BSA and 2% normal goat serum for 30 min, incubated with affinity-purified *Drosophila* AChE antibodies diluted 1:1000 in PBT plus 0.4% BSA overnight at 4°C, and returned to room temperature. After 1 h of incubation and three washes with PBT, the embryos were incubated for 2 h in a rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:250 in PBT containing 0.4% BSA and 2% normal goat serum, washed three times with PBT, and transferred to 50% glycerol, 1% *n*-propyl gallate in PBS for mounting on slides under a coverslip. Processed embryos were observed on a Zeiss Axioplan microscope by confocal imaging with a Bio-Rad MRC600 (Richmond, CA) system using an argon laser.

RESULTS

Rescue of *Ace* Lethals with Wild-type GPI-AChE Minigene Defines a Threshold for Development and Adult Viability

A large number of *Ace* alleles have been generated by several laboratories, and we examined six alleles assigned as null by genetic criteria and by the lack of

visible AChE histochemical staining in tissue cryosections (Hall and Kankel, 1976; Greenspan *et al.*, 1980). Animals homozygous for these lethal alleles die as pharate first instar larvae (L1). All six alleles were rescued by the GPI-AChE minigene, but these crosses suggested two allelic classes: true nulls with no AChE activity and severe hypomorphs with a residual activity of <5% of normal. This was confirmed by collecting large numbers of homozygous mutant embryos and assessing their AChE content by ND-PAGE and histochemical detection. Embryos homozygous for severely hypomorphic alleles had detectable AChE activity while the homozygous null embryos had none (Incardona and Rosenberry, unpublished data).

The *Ace* minigene encoding GPI-AChE (Figure 1; Hoffmann *et al.*, 1992) was constructed of 1.5 kb of 5' genomic DNA from the *Ace* region fused to a full length *Ace* cDNA. The size of the gene was reduced from over 37 kb to 5.8 kb by eliminating nine introns, making it more manageable for transformation and mutagenesis. This GPI-AChE minigene rescued (severely hypomorphic) lethal alleles by providing 20–30% of the normal AChE activity and produced an expression pattern essentially identical to that of AChE in wild-type flies at a crude level of analysis (Hoffmann *et al.* 1992). In this report we confirm these results and expand upon them by examination of both classes of *Ace* alleles with a wider range of GPI-AChE expression levels. Three X-linked lines (G1, G2, and G3) and four lines on the second chromosome (G4, G5,

Figure 1. (A) Schematic representation of the *Ace* minigene construct. The 5.8-kb *Ace* minigene was inserted into the *Xho*I site of pCaSpeR-4. The TM-AChE coding sequence was subcloned into the minigene via the *Ksp*I and *Asp*718 sites. pCaSpeR-4 also contains the wild-type *white* minigene for identification of transformants; progeny of injected white-eyed flies may have eye colors ranging from pale yellow to near wild-type red. (B) C-terminal peptide sequences of the constructs. WT, the signal peptide of GPI-AChE that is exchanged for the GPI; TM, the transmembrane and cytoplasmic (italicized) domains of the AChE/HSV gC fusion TM-AChE.

G6, and G7) were tested for their ability to rescue many combinations of *Ace* alleles. The rescue crosses included heteroallelic combinations of nulls and severe hypomorphs with each other and in combination with *Df(3R)l26d*, a deletion that removes *Ace* and six other complementation groups in the 87E region (Hiliker *et al.*, 1980). In most cases the crosses utilized a *y w* X chromosome and a *TM3 Ser y+* balancer to identify rescued animals at various stages of development by the color of the cuticle. The data from these crosses are summarized in Table 1. The three X-linked lines behaved very similarly, rescuing both nulls and severe hypomorphs with one copy of transgene. Females with one copy of the transgene typically expressed about 20% of the AChE activity in wild-type (*y w*) flies and hemizygous males expressed about 30%. Females with two copies showed a linear dosage effect and expressed a level of 40%. The higher level of expression in males was most likely due to the dosage compensation effect that is frequently observed when autosomal genes are transposed to the X chromosome (reviewed by Lucchesi and Manning, 1987). The second chromosome lines G4 and G5 were unable to rescue null alleles with two copies of transgene. However, for line G4 it was noted that a significant number of homozygous pupae completed metamorphosis but failed to eclose. The lethal phase thus was shifted from pharate L1 to pharate adults. Pharate adults that were dissected out of their pupal cases and found to be still

viable by weak movements and twitches were subsequently assayed for AChE activity. These animals had AChE activity of $9.0 \pm 0.1\%$ normal, indicating a level of activity that was sufficient for nearly complete development but not for viability of the adult. In high density cultures the "rescued" pupae were smaller than pupae carrying the balancer, and as larvae, did not feed as vigorously and tended to leave the food and pupariate several days later than the heterozygous siblings. The G4 line was able to rescue a heteroallelic combination of severe hypomorphs (*lm38/l26*), producing viable adults with about 12% of normal activity that could reproduce and be kept as a stock. These results indicate the following: 1) *l26* and *lm38* combined produce 3% of wild-type activity, an amount typical for the other *Ace* severe hypomorphs (Incardona and Rosenberry, unpublished data); and 2) adult lethality ensues at a sharp and relatively low threshold of AChE activity.

The gross phenotype of adult flies rescued by the GPI-AChE minigene appeared to be nearly normal. They were fertile, could be kept as a stock, and seemed generally vigorous. However, a small percentage of these flies could be induced to undergo paralysis by a mechanical shock that involved a 10-s pulse on a Vortex mixer at maximum speed (Ganetzky and Wu, 1982). Flies with this bang-sensitive phenotype typically responded to this treatment with a burst of rapid, uncoordinated motor activity followed by paralysis,

Table 1. Rescue with GPI-AChE transgene

Transgene	Nulls			Severe hypomorphs	
	H36/Df	H89/Df	B21-5/Df	lm38/Df	lm38/l26
G1 (X) ^a	+ (394) ^b	+ (996)	+ (810)	+ (704)	+ (151)
G2 (X)	+ (260)		+ (548)		
G3 (X)	+ (473)	+ (531)		+ (837)	
G4 (II)		– (341)			+ (417)
G5 (II)		– (868)			
G6/G7 (II)				+ (579)	

Genotype	% of normal AChE level ^c	Phenotype ^d
yw; G4; H89/Df	9.0 ± 0.1 (3)	Pharate adult death
yw; G4; lm38/l26	11.9 ± 0.2 (3)	Viable; occasional BS
G1/+; B21-5/Df ♀	20.7 ± 2.2 (4)	Viable; ND
G1/G1; B21-5/Df ♀	41.8 ± 4.4 (6)	Viable; ND
G1; B21-5/Df ♂	30.5 ± 2.1 (2)	Viable; ND
G3/+; lm38/Df ♀	ND	Viable; occasional BS
G3/G3; H89/Df ♀	40.7 ± 1.5 (4)	Viable; rare BS
G3; H89/Df ♂	28.2 ± 2.9 (3)	Viable; occasional BS

^a parentheses denote the chromosome carrying the insertion.

^b + denotes complete rescue with at least one copy of transgene; – indicates that no adult animals were rescued; parentheses denote total progeny counted.

^c mean and standard error, with number of animals assayed in parenthesis.

^d BS denotes bang sensitivity (see text); ND, not determined.

after which they recovered in about 30 s; wild-type flies were unaffected by vortexing. Flies that carry a semi-lethal hypomorphic allele *lm35* in combination with *Df(3R)l26d* had only 20% of normal AChE activity but were not bang sensitive. This suggests that limited genomic sequences or the lack of introns in the minigene construct may prevent proper expression in some subset of neurons that normally expresses AChE and that bang sensitivity is not simply related to the overall level of AChE expression. This interpretation is supported by the finding that expression from the minigene produces less AChE immunoreactivity in a single area of neuropil called the lamina (described below). Some degree of bang sensitivity was observed for all minigene lines with both GPI- and TM-AChE (see below), and with a secreted AChE construct (Incardona and Rosenberry, unpublished data). Moreover, the percentage of bang-sensitive flies decreased with more copies of transgene insert. These findings make it unlikely that the phenotype results from insertion of the transgenes into known bang-sensitive loci, of which there are at least four in *Drosophila*: *bang-sensitive*, *bang-senseless*, *easily shocked* (reviewed by Hall, 1982), and the gene for (Na⁺,K⁺)ATPase α subunit (Schubiger *et al.*, 1994). Because the bang-sensitivity is related to all types of *Ace* minigene and not specifically to either GPI-anchored or TM-AChE, we have not explored its basis further. However, data

concerning another component of the cholinergic system also suggest that the bang sensitivity may be related to control elements in the minigene construct: molecular dissection of the 5'-flanking region of the gene for *Drosophila* choline acetyltransferase (the acetylcholine biosynthetic enzyme) showed that discrete segments controlled expression in subsets of neuropil (Kitamoto *et al.*, 1992).

Transmembrane AChE Can Rescue Null Alleles with an Efficiency Nearly Equal to the Wild-type Minigene

The strategy for rescue crosses with the TM-AChE minigene was identical to that described for the GPI-AChE minigene and utilized the same sets of both severely hypomorphic and null alleles, as summarized in Table 2. Six X-linked inserts and one second chromosome insert were tested. In crosses combining null alleles and the deletion, the majority of the animals that eclosed were females with two copies of transgene and AChE levels of 13–16% of normal. These animals showed a clear phenotypic difference from rescued flies expressing a comparable level of GPI-AChE. Eclosed females that were scored appeared to be weak and all were very sensitive to mechanical shock, sometimes undergoing spontaneous reversible paralysis. These females usually died several days

Table 2. Rescue with TM-AChE transgene

Transgene	Nulls			Severe hypomorphs	
	H36/Df	H89/Df	B21-5/Df	lm38/Df	lm38/l26
T1 (X) ^a		– (80) ^b			– (82)
T2 (X)		– (101)			
T3 (X)			± (424)	± (114)	± (113)
T4 (X)		± (781)			
T5 (X)		± (147)			± (79)
T6 (X)		± (176)			
T3; T7 (X, II)		± (1185)			
T3.5 (rec)	+ (693)	+ (483)	+ (354)		

Genotype	% of normal AChE level ^c	Phenotype ^d
T3/+; B21-5/Df ♀	7.4 ± 0.3 (2)	Pharate adult death
T3/T3; B21-5/Df ♀	13.4 ± 1.5 (3)	Pharate adult death; high BS, early death
T3/T3; l26/lm38 ♀	15.1 ± 1.8 (3)	Viable; frequent BS
T5/T5; H89/Df ♀	16.3 ± 0.7 (4)	Pharate adult death; high BS, early death
T5/T5; l26/lm38 ♀	19.6 ± 1.5 (3)	Viable; frequent BS
T3; T7; H89/Df ♀	27.7 ± 3.7 (8)	Viable; occasional BS; uncoordinated
T3.5; H36/Df ♂	18.7 ± 0.8 (2)	Viable; weak; frequent BS; uncoordinated
T3.5/T3.5; H36/Df ♀	28.4 ± 1.0 (3)	Viable; occasional BS; uncoordinated

^a parentheses denote the chromosome carrying the insertion.

^b + denotes complete rescue; ± indicates rescue of females with two copies of transgene only; – indicates that no adult animals were rescued; parentheses denote total progeny counted.

^c mean and standard error, with number of animals assayed in parenthesis.

^d BS denotes bang sensitivity (see text).

after eclosion. An equal number of eclosed females bearing two copies of transgene were found drowned in the food within 30 min of eclosion but were not included in the score. Males with a single copy of transgene and AChE levels of only 7–10% died as pharate adults, were trapped halfway out of the puparium, or were only able to open the operculum. In these respects, inserts T3, T4, T5, and T6 showed identical phenotypes. However, when these transgene inserts were used in rescue crosses with the severe hypomorphs *l26* and *lm38*, most animals were able to eclose and the phenotype was much less severe. These rescued males and females were easily maintained as stocks suggesting relatively normal fertility, fecundity, and ability to mate.

To increase the dosage of transgene, we repeated the rescue crosses using a combination of X and second chromosome inserts (T3 and T7, respectively) and a recombinant X chromosome (T3.5) bearing both the T3 and T5 inserts. As indicated in Table 2, higher doses of TM-AChE transgene were able to rescue all three null alleles with a range of efficiencies similar to those observed for the GPI-AChE transgene. Animals rescued with either the T3/T7 combination or the T3.5 chromosome were fertile and easily maintained as stocks and had enzyme levels of about 30%. In contrast to GPI-AChE, the anchor of which is completely cleaved by PIPLC (Haas *et al.*, 1988; Fournier *et al.*, 1988), all of this AChE activity was resistant to PIPLC and thus derived from TM-AChE (Incardona and Rosenberry, 1996; our unpublished results). Although a small percentage of these animals were also bang-sensitive, the frequency of bang-sensitivity was no higher than that of flies rescued by the GPI-AChE

minigene. Therefore, when enzyme activity levels provided by TM-AChE were closer to the highest levels achieved with the GPI-AChE transgene, the phenotypes of the two became more similar. However, these TM-AChE-expressing animals appeared to have a lifespan that was less than that of flies rescued by the GPI-AChE transgene.

We compared the lifespan of cohorts of flies rescued by TM- and GPI-AChE transgenes. In the population represented in Figure 2, 100% of flies of both sexes rescued by the GPI-AChE minigene were alive 3 days after eclosion, while 80% of females rescued by TM-AChE were surviving. By 12 days after eclosion, 92% and 86% of females and males rescued by GPI-AChE, respectively, were alive, while only 38% of females rescued by TM-AChE were alive. The mean half-life of the female population rescued by TM-AChE was found to be 7.3 ± 1.5 days from five cohorts. The half-life of populations rescued by GPI-AChE was >20 days. Flies expressing TM-AChE were frequently observed trapped alive in the food or with their wings adhering to the sides of the vials. We thus suspected that the reduced lifespan was probably secondary to either weakness or uncoordination rather than a "drop-dead" phenotype observed in a *Drosophila* neurodegenerative mutant (Buchanan and Benzer, 1993).

Flies Expressing Transmembrane AChE Have Reduced Locomotor Behavior and Are Uncoordinated

We used several behavioral tests to analyze further the phenotype of flies expressing TM-AChE (Table 3). Using a standard assay for locomotor activity in *Drosophila*

Figure 2. Analysis of the lifespan of rescued flies. Females expressing TM-AChE (filled circles), females expressing GPI-AChE (open circles), and males expressing GPI-AChE (open squares) were collected within 24 h of eclosion and stored at a density of about 20 flies per vial. Flies were counted every 2–3 days and transferred to a fresh vial at each counting. The data shown represent cohorts of 54, 55, and 57 animals, respectively. Over 100 total animals of each genotype were used to measure a mean population half-life. TM-AChE flies were rescued with the T3/T7 combination of inserts, and GPI-AChE flies were rescued with the G3 insert. See Tables 1 and 2 for associated AChE activities.

Table 3. Behavioral tests

Genotype ^a	Locomotor activity score ^b	Climbing score ^b	Grooming % dusty ^c
Wild-type ♀	64 ± 4	3.7 ± 0.7	0 (N = 66)
GPI-AChE ♀	44 ± 3	3.7 ± 0.5	8 (N = 37)
GPI-AChE ♂	40 ± 3	4.0 ± 0.7	11 (N = 44)
TM-AChE ♀	25 ± 5 ^d	0.8 ± 0.3 ^e	77 (N = 73)

^a genotypes are as listed for the G3 and T3; T7 transgenes in Tables 1 and 2.

^b locomotor activity and climbing are defined under MATERIALS AND METHODS; values are the mean and standard error (N = 15–20).

^c percentage of animals scored as ungroomed (dusty).

^d $p \leq 0.005$ compared with GPI-AChE females, $p < 0.03$ compared with GPI-AChE males.

^e $p < 0.005$ compared with GPI-AChE or wild type.

ila (Connolly 1966; Hall *et al.*, 1980), we compared animals rescued by either GPI- or TM-AChE transgenes that expressed similar levels of AChE activity (see above). Although not directly measuring coordination, this assay reflects the initiation of spontaneous activity and the general speed at which an individual fly moves. Flies rescued by the GPI-AChE transgene had a mean locomotor activity of 44 ± 3 for females and 40 ± 3 for males, while animals expressing TM-AChE had a mean locomotor activity of 25 ± 5 . This difference was statistically significant. Rather than not moving at all, the animals expressing TM-AChE appeared to be sluggish. Locomotor activity may depend on AChE levels in general, particularly that present during pupal development (Hall *et al.*, 1980). The reduced activity of flies expressing TM-AChE therefore suggests either ineffective AChE localization or possibly defective development of the CNS during metamorphosis.

In addition to general locomotor activity, we were also able to observe a behavior more specifically reflective of coordination: the ability of animals to climb and move between the floor and roof of the observation chamber. Flies expressing GPI-AChE were found to transfer between the floor and the roof an average of 3.7 ± 0.5 times for females and 4.0 ± 0.7 for males during 5 min of observation. Females expressing TM-AChE at a comparable level transferred successfully only 0.8 ± 0.3 times in the same period. This difference was also found to be statistically significant compared with both wild type (*y w*) and flies rescued by the GPI-AChE transgene. It was noted that the animals expressing TM-AChE frequently attempted to climb, but they would usually fail. The parental *y w* stock had a general locomotor activity of 64 ± 4 with 3.7 ± 0.7 transfers. The difference between the locomotor activity of the parental stock and GPI-AChE-rescued flies

was significant, but the difference in the number of transfers was not.

To assess the ability to coordinate a more complicated activity, we used a recently developed assay of grooming behavior. *Drosophila* clean themselves using a stereotypical sequence of leg and body movements that has been well described (Svebenyi, 1969). Phillis *et al.* (1993) developed a simple grooming assay for *Drosophila* and isolated mutants defective in grooming behavior based upon it; these mutants included two classes that were defective in either motor coordination or mechanosensation. These authors also demonstrated that other known *Drosophila* neurological mutants that have been described as uncoordinated failed to perform well in the assay. When male flies rescued by the GPI-AChE transgene were assayed, 11% were scored as dusty, while 8% of females were scored as dusty. TM-AChE-rescued females expressing a similar level of AChE activity were poor groomers, with 77% scored as dusty. The TM-AChE-expressing flies had difficulty grooming the wings in particular, but in general had some dust remaining on all body parts. Phillis *et al.* (1993) defined normal grooming as <25% scored as dusty, reduced grooming ability as 50–90% scored as dusty, and abnormal as >90% scored as dusty. The results we obtained with TM-AChE-expressing flies in this assay therefore indicate that they are uncoordinated, although not grossly.

Immunocytochemical Localization of TM-AChE and GPI-AChE in Brains of Rescued Animals Appears Identical at the Light Microscopic Level

The slightly higher threshold required for rescue by TM-AChE and the behavioral defects observed in adults expressing higher levels suggested that TM-AChE may be mislocalized. Although these animals had AChE activity in homogenates sufficient for normal behavior, they behaved as if they had a lower effective level. To compare the localization of GPI-AChE and TM-AChE in rescued adults, we performed immunocytochemistry using affinity-purified polyclonal antibodies specific for the ectodomain of *Drosophila* AChE. In general there are three major histological components to the insect brain: a cortex or “perikaryal rind” of neuronal cell bodies, synaptic neuropil comprising the arborizations and interconnections of neurons, and tract neuropil that consists of the major axon bundles and is analogous to vertebrate white matter. Since AChE is a synaptic protein it was found predominantly in synaptic neuropil, but significant immunoreactivity was also found in the cortex and on some but not all major fiber tracts (Figure 3), consistent with previous reports (e.g. Wolfgang and Forte, 1989). The pattern of immunoreactivity observed in flies rescued by GPI-AChE was essentially identical to that observed for a true wild-type fly

(Figure 3, A and G, respectively) except that the immunoreactivity as a whole was reduced; the rescued fly in Figure 3A expressed about 40% of the normal activity. However, immunoreactivity in the lamina cortex seemed to be disproportionately reduced. Extensive analysis of serial cryostat sections of many rescued flies found no other obvious difference in the expression pattern of AChE in wild-type flies and flies with *Ace* null alleles after rescue by the GPI-AChE minigene (our unpublished results), suggesting that the 1.5-kb genomic segment of the minigene construct contains most of the necessary control elements.

Further analyses of serial sections of all major functional units of synaptic neuropil showed no obvious differences in the pattern of AChE immunoreactivity between flies rescued with the GPI-AChE or TM-AChE minigenes (Figure 3B). We examined all of the following regions of neuropil: the optic lobe, including the lamina, medulla, lobula, and lobula plate; the antennal lobe; the mushroom bodies; the central complex, including the fan-shaped body, the elipsoid body, the protocerebral bridge, and the noduli; and the subesophageal and thoracic ganglia. We focused more closely on the visual system because the optic lobe is the best characterized region of the fly CNS in terms of cell types and cellular morphology (e.g. Fischbach and Dittrich, 1989; Meinertzhagen and O'Neil, 1991) and development (reviewed by Hansen and Meinertzhagen, 1993), and any gross mislocalization of TM-AChE should be apparent in the intricate pattern of the repeating elements. However, we also observed no differences in brain regions that are more complicated and less well-characterized. This is illustrated in Figure 3, C–F, for the central complex, a brain region thought to be involved in the higher control of motor function (Strauss and Heisenberg, 1993), and of particular interest due to the uncoordinated phenotype observed with TM-AChE. Both minigenes gave strong labeling of the noduli, a region that is densely packed with synapses (Strausfeld, 1976). Rescued flies with lower levels of TM-AChE and a more severe phenotype also did not show an obvious difference in the localization of AChE immunoreactivity in the CNS, and immunocytochemistry on serial cryostat sections through the thoracic ganglion again showed no apparent differences between wild-type flies and flies rescued by either the GPI-AChE or TM-AChE minigenes (our unpublished observations).

Immunoelectron Microscopic Localization of GPI- and TM-AChE Shows No Apparent Differences

Although light level immunocytochemistry showed no obvious difference in the distributions of GPI- and TM-AChE, it is possible that a subtle difference could not be resolved within the dense tangle of synaptic neuropil. Therefore, we examined flies expressing

GPI-AChE or TM-AChE by immunoelectron microscopy with a pre-embedding peroxidase method that produces a precipitate similar to that in the cryostat sections. An essentially identical technique has been used to show polarized expression of the GPI-anchored F3/F11 cell adhesion molecule in mouse cerebellum (Faivre-Sarrailh *et al.*, 1992). Although the lamina exhibits low levels of AChE immunoreactivity (Figure 3), it was examined in control counterstained sections because it is the only neuropil of flies for which synaptic structures have been well studied (Meinertzhagen and O'Neil, 1991, and references therein). Analysis of these sections demonstrated the preservation of all characterized elements of photoreceptor terminals, including pre- and post-synaptic specializations, capitate projections, synaptic vesicles, and septate junctions, and similar structures and others such as dense core vesicles were observed in deeper areas of optic lobe neuropil that are normally strongly positive for AChE (our unpublished observations). Parallel vibratome sections from a wild-type animal labeled with affinity-purified anti-AChE antibodies revealed that immunoreactivity was not strictly associated with synapses and occurred largely extra-synaptically. Many nonsynaptic neuronal elements were labeled, in particular many small, clear profiles (Figure 4A). Synaptic vesicle-containing profiles with AChE immunoreactivity were found infrequently, relative to labeled profiles that were devoid of vesicles. Immunoreactivity also was observed in small, elongated patches at points of contact between neighboring cells. However, all AChE immunoreactivity appeared to be associated with the plasma membrane in the extracellular space, and intracellular structures were not labeled except in cases that were clearly associated with artifacts obtained near the surface of the vibratome sections (Landis, 1985). Comparison of wild-type flies and flies with *Ace* null alleles after rescue by the GPI-AChE or TM-AChE minigenes (Figure 4B) showed no differences except for a lower level of immunoreactivity from the minigenes. TM-AChE immunoreactivity was found in patterns similar to those observed for GPI-AChE, including the elongated patches between cells. Furthermore, TM-AChE immunoreactivity also was not associated with any intracellular organelles except in artifactual situations near the surface of sections.

In cortical regions of the brain, we did observe immunoreactivity associated with cell bodies on plasma, endoplasmic reticulum, and Golgi membranes for both GPI- and TM-AChE, but there was no apparent difference in the extent of labeling (our unpublished results). We also observed labeling of the nuclear membranes for both AChEs, but this was probably artifactual.

Figure 3. Light-level immunocytochemical localization of GPI- and TM-AChE in the adult brain. Cryostat sections (10 μ m) were prepared and stained with affinity-purified anti-*Drosophila* AChE antibodies as described in MATERIALS AND METHODS; immunoreactivity is indicated by a purple precipitate. The orange-brown color of the retina in transgenic flies is the natural color of eye pigments provided by the *white+* transposon marker and absent in the parental *yw* strain (Figure 3, compare A and B to G). (A, C, and E) Sections from flies of genotype G3;H89/Df rescued by the GPI-AChE minigene; (B, D, and F) sections from flies of genotype T3; T7; H89/Df rescued by the TM-AChE minigene; (G) a section from the wild-type strain (*yw*) used as a host for the transgenes. (A) Distribution of GPI-AChE in the optic lobe. Synaptic neuropil is strongly labeled. Major fiber tracts that label weakly are the first optic chiasm (x), the second optic chiasm (asterisk), and Cucatti's bundle (Cu, curved arrow), which carries many contralateral axons of tangential neurons into the serpentine layer of the

GPI- and TM-AChE Are Sorted Identically in Epithelial Tissues of the Adult and the Embryo

Immunocytochemistry on sections of the thorax with affinity-purified AChE antibodies revealed that AChE is normally expressed in the cardia and the proximal portion of the midgut (Figure 5). This immunoreactivity was observed in both wild-type flies (our unpublished results) and in flies rescued by the GPI-AChE or TM-AChE minigenes, indicating that it was not a result of inappropriate expression from the minigenes. The cardia of Dipteran flies is a specialized organ that lies at the junction of the esophagus and the midgut and produces the peritrophic matrix, a proteinaceous membrane that lines the intestine (reviewed by Richards and Richards, 1977). It has a three-walled structure consisting of an inner or esophageal wall, a recurrent wall, and an outer wall. King (1988) described six zones of the cardia based on ultrastructurally distinct epithelial cell types, all of which are highly polarized but individually specialized for secretion of either a particular component of the peritrophic matrix or cuticle. As shown in Figure 5B, GPI-AChE was localized exclusively in the apical microvilli of the cells of outer wall zones 4–6; these cells are derived from the midgut and secrete the peritrophic matrix. In contrast, the columnar cells of recurrent wall zones 1–3 showed unpolarized AChE immunoreactivity localized along the entire periphery of the cells in addition to the apical surface. These cells of the recurrent wall are derived from foregut and, like the cells lining the esophagus, secrete cuticle. The pattern of AChE immunoreactivity in flies expressing TM-AChE appeared identical to that of flies with GPI-AChE (Figure 5C). To demonstrate that the immunoreactivity was indeed specific for AChE and not due to a cross-reacting epitope, we performed AChE histochemistry with an AChE activity stain (Tago *et al.*, 1986) on parallel sections in the presence and absence of eserine, a specific AChE inhibitor. The pattern of deposition of AChE enzymatic reaction product in the cardia (Figure 5A) was very similar to that of the immunoreactivity, and the pattern was abolished by eserine. Hall and Kankel (1976) demonstrated eserine-resistant general esterase staining more distally in intestine, and their data suggest the presence of this activity in the cardia. However, this organ was not examined carefully in their study.

To investigate the sorting of GPI- and TM-AChE by additional types of polarized epithelia, flies were transformed with P element vectors carrying the GPI- or TM-AChE cDNAs under control of a heat shock promoter. The two constructs were expressed ectopically in all tissues of *Drosophila* embryos by subjecting a collection of transformed embryos at different developmental stages to a 1-h heat shock, and after a 5-h recovery period AChE was detected by indirect immunofluorescence and laser scanning confocal microscopy. As found in adult tissues, GPI-AChE (Figure 6A) and TM-AChE (Figure 6B) were sorted identically in polarized embryonic cells. Both were targeted to basolateral surfaces in the cuticle-secreting surface epithelium of embryos and to the apical surfaces associated with the lumina of internal organs like the salivary gland and midgut. Interestingly, GPI-AChE was sorted basolaterally in the hindgut epithelium, which, unlike midgut epithelium, secretes cuticle.

DISCUSSION

Data from cultured mammalian cells suggest that a primary function of GPI anchors is to direct the attached proteins to membrane subdomains (Brown and Rose, 1992; Cerneus *et al.*, 1993; Sargiacomo *et al.*, 1993). Despite similarities in the polarized sorting of membrane proteins in mammalian epithelial cells and neurons (reviewed by Kelly and Grote, 1993), it is unclear whether GPIs are widely recognized as a targeting signal. In studies of mammalian neurons in culture and in vivo, GPI-anchored proteins showed both strictly axonal and nonpolarized distributions (Dotti *et al.*, 1991; Faivre-Sarrailh *et al.*, 1992). Furthermore, studies on GPI-anchored proteins in cultured epithelial cells, while providing a wealth of new information on the polarized sorting of proteins and lipids, have not shed much light on how GPIs are involved in the functions of the proteins that they anchor. Our goals in this study were to express an AChE with a transmembrane peptide anchor (TM-AChE), assess whether it was delivered to the same subcellular location as that of wild-type GPI-AChE at the right time, and determine whether it would substitute functionally for GPI-AChE. Cloned DNA from the *Ace* locus provided the tools needed for efficient in vivo manipulation of AChE coding sequences and for expression of altered AChEs in the tissues where it

(Figure 3 cont.) medulla (me). There is differential labeling of the layers of the medulla; the solid arrow marks the strong labeling of layer 10 (Fischbach and Dittrich, 1989), while the unfilled arrow marks alternating strong and weak labeling of layers adjacent to the serpentine layer. The retina (re) shows only background immunoreactivity and the orange-brown pigment granules. The lamina neuropil (la) is normally weakly positive for AChE; however, the usually strong labeling of laminar cell bodies just beneath the retina is reduced in transgenic animals (compare to G). (B) The distribution of TM-AChE in the optic lobe. Arrows point out structures corresponding to those in panel A. (C) GPI-AChE immunoreactivity in elements of the central complex of a rescued animal. fb, fan-shaped body; eb, ellipsoid body. (D) TM-AChE immunoreactivity in the corresponding structures of a fly rescued by TM-AChE minigene. (E) GPI-AChE immunoreactivity in the noduli (no) of the central complex. (F) TM-AChE immunoreactivity in the noduli. (G) GPI-AChE immunoreactivity in the optic lobe of a wild-type (*yw*) animal from the parental strain used for P element transformation. Arrows point out structures corresponding to those in panel A. Scale bar, 50 μ m.

Figure 4. Immunoelectron microscopic localization of GPI- and TM-AChE in the optic lobe. Vibratome sections were labeled with affinity-purified *Drosophila* AChE rabbit antibodies, followed by biotinylated anti-rabbit IgG and avidin-peroxidase histochemistry. Lack of immunoreactivity in sections labeled with an irrelevant primary antiserum demonstrated the specificity of the AChE antibodies. (A) GPI-AChE immunoreactivity in the medulla of a wild-type (*yw*) fly. (B) TM-AChE immunoreactivity in the medulla of a fly of genotype *T3; T7; H89/Df*. Note circumferential extracellular labeling of small axonal fibers (curved arrow), and discrete patches of label at junctions between cells (short arrows). Scale bars, 0.5 μ m.

normally is expressed. Transgenic flies expressing either GPI-AChE or TM-AChE were generated using P element transposon vectors in genetic backgrounds that either completely lacked (null mutants) or had <5% (severe hypomorphs) of normal endogenous AChE.

Rescue crosses with a panel of wild-type GPI-AChE minigene lines gave AChE activities ranging from

9–40% of normal in the progeny and revealed the following. 1) A threshold level of GPI-AChE activity of only about 12% of normal was sufficient for complete development, adult viability, and normal behavior. (2) The G4 line that rescued severe hypomorphs with 12% of normal activity also shifted the lethal phase in nulls from pharate L1 to pharate adults with 9% of normal activity. Thus 9% was sufficient for nearly complete

Figure 5. Demonstration of GPI- and TM-AChE expression in the cardia. Cryostat sections (10 μ m) were prepared and AChE detected as described in MATERIALS AND METHODS. (A) Histochemical detection of GPI-AChE activity in the cardia of a true wild-type (*yw*) fly. Zones of the cardia are labeled according to the method of King (1988). Cells of zones 1–3 are derived embryonically from foregut and secrete a cuticular lining from their apical face. Cells from zones 4–6 are derived from the midgut and are contiguous with the proximal portion thereof, have a higher density of apical microvilli, and secrete the components of the peritrophic matrix into the

development but not for viability of the adult. (3) The level of GPI-AChE activity was proportional to the copy number of the transgene.

To construct TM-AChE, the sequence encoding the C-terminal signal peptide in the *Ace* cDNA was replaced with a segment encoding the transmembrane domain and small cytoplasmic tail of HSV-1 gC. We have shown that this TM-AChE has the same catalytic specificity as GPI-AChE and is delivered to the surface of cultured *Drosophila* S2 cells where it has a half-life essentially identical to that of GPI-AChE (Incardona and Rosenberry, 1996). Slightly higher levels of TM-AChE activity were required to rescue *Ace* null alleles (Table 2). Animals expressing about 15% of normal activity from a TM-AChE minigene showed an abnormal phenotype not observed in flies expressing a level of 12% of normal from a GPI-AChE minigene: most animals died shortly after eclosion. These animals did not appear to coordinate the suite of highly specialized motor programs that are performed during and immediately after eclosion, such as expansion and drying of the wings. When levels of TM-AChE were raised to about 30% of normal, rescue proceeded with an efficiency equal to that of GPI-AChE, the eclosion phenotype abated, and the animals in general behaved similarly to animals expressing a comparable level of GPI-AChE. However, it was noted that even these flies expressing a higher level of TM-AChE had a lifespan reduced from several weeks to <10 days, probably secondary to uncoordination (Figure 2). Using two assays we showed quantitatively that the TM-AChE-rescued flies do in fact have motor defects and are uncoordinated (Table 3). An assay of general locomotor behavior demonstrated that flies expressing TM-AChE had reduced walking activity and difficulty climbing. A grooming assay showed that these same animals had difficulty in coordinating this complicated stereotypical behavior.

Our immunocytochemical studies showed no differences in the cellular localization of GPI-AChE and TM-AChE, raising the question of whether sorting signals identified in mammalian proteins are read correctly in *Drosophila*. One study has indicated that a mammalian basolateral targeting signal is recognized in some cells. Shiel and Caplan (1995) used heat shock vectors to express two forms of human placental alkaline phosphatase in *Drosophila* embryos. They

(Figure 5 cont.) narrow lumen of the organ. The esophagus passes through the center of the cardia. The asterisk marks AChE histochemical activity in the thoracic ganglion. (B) GPI-AChE immunoreactivity in a fly of genotype G3; *H89/Df* rescued by the GPI-AChE minigene. Arrowheads mark the basal and lateral surfaces of cells and the curved arrows mark the apical surfaces. The immunoreactivity at the base of zone 5 is associated with muscle fibers that ensheath the cardia rather than the zone 5 cells themselves. (C) TM-AChE immunoreactivity in the cardia of a fly of genotype T3; T7; *H89/Df* rescued by the TM-AChE minigene. Scale bar, 50 μ m.

Figure 6. Sorting of GPI- and TM-AChE in epithelial tissues of the embryo. AChE immunoreactivity was detected in embryos expressing GPI-AChE and TM-AChE ectopically under heat shock control as described in MATERIALS AND METHODS. (A) GPI-AChE immunoreactivity in an hsE18 embryo. Arrowheads mark basolateral surfaces and arrows mark apical surfaces. The curved arrow marks the lumen of the midgut (mg). Left inset shows immunoreactivity confined to the apical surfaces of the salivary gland (sg) in the same embryo; the lumen

showed by confocal microscopy that GPI-anchored alkaline phosphatase was sorted to the apical surfaces of polarized epithelial tissues of internal organs, while a transmembrane chimera that contained a dominant basolateral sorting signal from vesicular stomatitis virus glycoprotein protein was sorted to the respective basolateral surfaces. This pattern was consistent with the sorting of these two proteins in MDCK cells (Brown *et al.*, 1989; Thomas *et al.*, 1993). However, both of these proteins were sorted basolaterally in the cuticle-secreting surface epithelium of the embryo. In other insect species endogenous GPI-anchored alkaline phosphatase also was sorted to the apical microvilli of the midgut (Azuma and Eguchi, 1989) but was nonpolarized in the surface epithelium (Chang *et al.*, 1993). The localization of GPI-AChE in epithelia in our study are consistent with these results: GPI-AChE was apically targeted in some internal organs such as the salivary gland (Figure 6A) and the midgut-derived region of the cardia (Figure 5B), but basolaterally targeted or nonpolarized in cuticle-secreting epithelia of the foregut, hindgut, and surface. In TM-AChE, the transmembrane domain and the 11-residue cytoplasmic tail from HSV-1 gC (Figure 1) contain none of the basolateral sorting signals identified in vesicular stomatitis virus glycoprotein or other proteins (e.g. Matter *et al.*, 1992; Thomas *et al.*, 1993), and the identical localization of TM- and GPI-AChE that we observed demonstrates that these gC domains do not contain a dominant basolateral signal recognized by insect cells. HSV-1 gC itself is sorted to the basolateral surface in herpes-infected epithelial cells (Srivinas *et al.*, 1986), but it is unknown whether this is an inherent property of gC or if other virally encoded factors are necessary.

Insect neurons are functionally and morphologically polarized, although not with respect to the cell body, and they tend to be more complicated than their vertebrate counterparts with some processes having both pre- and post-synaptic elements (Ramón y Cajal, 1937; Strausfeld, 1976; Fischbach and Dittrich, 1989). Despite the complexity of *Drosophila* synaptic neuropil, light-level immunocytochemistry of the CNS suggested that TM- and GPI-AChE are also sorted identically by neurons. AChE immunoreactivity from both constructs is conserved in intricate patterns such as that of the central complex and the columnar elements in the optic lobe (Figure 3). The morphologies of individual neurons that contribute to the patterns of the optic lobe have been well characterized by Golgi studies (Fischbach and Dittrich, 1989). Changes in the distribution of AChE on processes of these cells from

"axonal" to "dendritic," for example, would undoubtedly alter the overall pattern. On the other hand, both GPI- and TM-AChE were observed on neuronal cell bodies as well as on processes in both synaptic neuropil and tracts, indicating that AChE expression in fly neurons may be normally nonpolarized.

The CNS distribution of TM- and GPI-AChE also were compared at the electron microscopic level (Figure 4), but again no differences were found. Both TM- and GPI-AChE appeared to be largely extrasynaptic and not associated with any specific membrane domains such as coated pits or caveolae. The discrete elongated patches of immunoreactivity observed for both GPI- and TM-AChE are intriguing, but they do not appear to represent any characterized membrane or cytoskeleton-associated structures. Technical limitations involving inconsistencies of antibody penetration in vibratome sections made quantitative comparisons in identical brain regions practically impossible. It is conceivable that there is a very subtle redistribution of TM-AChE on a small subset of neurons, observation of which has escaped the relatively small number of synaptic elements seen in our study. However, there was no gross defect in the distribution of TM-AChE, as may have been predicted by the data from mammalian cells. Neither GPI-AChE nor TM-AChE appear to be targeted to any specific membrane subdomain. Unfortunately, we have not identified the cellular basis for the abnormal behavioral phenotype of flies expressing TM-AChE.

Our observations that TM-AChE and GPI-AChE are localized identically indicate that in *Drosophila* cells the GPI anchor does not play a major role in targeting AChE. Furthermore, we found no evidence of biochemical and morphological features typical of GPI-anchored proteins in MDCK cells. Both TM-AChE and GPI-AChE were completely soluble in cold Triton X-100 extracts of fly heads or cultured *Drosophila* S2 cells (Incardona and Rosenberry, 1996), conditions under which membrane microdomains rich in GPI-anchored proteins resist solubilization. No membrane structures clearly resembling caveolae were obvious in our study of *Drosophila* brain, in a detailed ultrastructural study of the *Drosophila* cardia (King, 1988), or in a comprehensive electron microscopic survey of the development of cellular junctions in tissues of the *Drosophila* embryo (Tepass and Hartenstein, 1994). AChE in primary cultures of CNS neurons from rescued third instar larvae showed identical clustered patterns for GPI- and TM-AChE by both immunofluorescence and immunogold electron microscopy (Incardona, 1995). However, the clusters were not associated with any obvious membrane specializations nor were they dispersed by cholesterol-binding antibiotics that disrupt caveolae. The GPI anchor must be necessary for some subtler aspect of AChE physiology, as indicated by the behavioral phenotype obtained with

(Figure 6 cont.) is marked with an asterisk. Right inset shows the hindgut (hg) of the same embryo. (B) TM-AChE immunoreactivity in an hsTM embryo of a slightly earlier stage than that in panel A. Symbols are as described above. Inset shows the developing midgut in the same embryo. Scale bar, 50 μ m.

TM-AChE expression. Some form of membrane attachment is essential for AChE function, as an *Ace* minigene expressing a secreted AChE construct did not permit rescue (Incardona and Rosenberry, unpublished data). The low levels of AChE activity in animals with up to six copies of this minigene indicated that soluble AChE is rapidly degraded *in vivo*.

It is possible that cleavage of GPI-AChE by an endogenous phospholipase occurs in the remodeling of some larval neurons during metamorphosis and that altered metabolism of acetylcholine in TM-AChE larvae or pupae would produce subtle defects in adult motor systems. Ablation studies in the hawkmoth *Manduca* have suggested that adult coordination depends largely on premotor interneurons that have larval origins (Truman and Booker, 1986), and it is notable that when animals carrying temperature-sensitive combinations of *Ace* alleles were subjected to nonpermissive temperature during metamorphosis, severe locomotor defects were observed in the emerging adults (Hall *et al.*, 1980). Future studies on the pupal development of animals expressing TM-AChE may prove insightful. Despite difficulties in experimental design, our data suggest that hypothetical functions of GPI anchors other than protein sorting should be pursued in a wider variety of systems and cell types with the same attention that the sorting function has received.

ACKNOWLEDGMENTS

We extend our gratitude to Didier Fournier for providing the *Ace* minigene construct and for discussion of unpublished data, and to Jeff Hall and Arthur Chovnick for providing their *Ace* alleles. We thank Story Landis and members of her lab for loaning reagents and answering myriad questions on fixation, immunocytochemistry, light and electron microscopy, and photographic techniques; Ruth Siegel for advice and use of her cryostat and other equipment; Dennis Landis for guidance in electron microscopy methods and interpretation of electron micrographs; Joe Pollack and Kellye Petonovich for excellent assistance with the electron microscopy; Bill Wolfgang and Mike Forte for discussion of immunocytochemical techniques and for providing anti-*Drosophila* AChE monoclonal antibodies used in early phases of this work; Ian Meinertzhagen for discussion of and advice on cryosectioning and electron microscopic methods in insects; Cathy Doller for vibratome lessons; Beth Albrecht for lessons on *Drosophila* transformation techniques; Karen Gustashaw for help with the confocal microscopy; Michael Caplan for sharing unpublished results; and Peter Harte, Helen Salz, and Marcelo Jacobs-Lorena for genetic counseling. This work was supported by grant NS-16577 from the National Institutes of Health and grants from the Muscular Dystrophy Association to T.L.R., and a Medical Scientist Training Program predoctoral fellowship from the National Institute of General Medical Sciences to J.P.I. (grant 2T32 GM-07250).

REFERENCES

Ashburner, M. (1989). *Drosophila: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Azuma, M., and Eguchi, M. (1989). Discrete localization of distinct alkaline phosphatase isozymes in the cell surface of silkworm midgut epithelium. *J. Exp. Zool.* 251, 108–112.

Barth, A., Muller-Taubenberger, A., Taranto, P., and Gerisch, G. (1994). Replacement of the phospholipid-anchor in the contact site A glycoprotein of *D. discoideum* by a transmembrane region does not impede cell adhesion but reduces time on the cell surface. *J. Cell Biol.* 124, 205–215.

Brown, D. (1993). The tyrosine kinase connection: how GPI-anchored proteins activate T cells. *Curr. Opin. Immunol.* 5, 349–354.

Brown, D.A., Crise, B., and Rose, J.K. (1989). Mechanism of membrane anchoring affects polarized expression of two proteins in MDCK cells. *Science* 245, 1499–1501.

Brown, D.A., and Rose, J.K. (1992). Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* 68, 533–544.

Buchanan, R.L., and Benzer, S. (1993). Defective glia in the *Drosophila* brain degeneration mutant *drop-dead*. *Neuron* 10, 839–850.

Bunch, T.A., and Brower, D.L. (1993). *Drosophila* cell adhesion molecules. *Curr. Top. Dev. Biol.* 28, 81–123.

Cerneus, D.P., Ueffing, E., Posthuma, G., Strous, G.J., and van der Ende, A. (1993). Detergent insolubility of alkaline phosphatase during biosynthetic transport and endocytosis. *J. Biol. Chem.* 268, 3150–3155.

Chang, W.S., Zachow, K.R., and Bentley, D. (1993). Expression of epithelial alkaline phosphatase in segmentally iterated bands during grasshopper limb morphogenesis. *Development* 118, 651–663.

Chase, B.A., and Kankel, D.R. (1988). On the role of normal acetylcholine metabolism in the formation and maintenance of the *Drosophila* nervous system. *Dev. Biol.* 125, 361–380.

Connolly, K. (1966). Locomotor activity in *Drosophila*. II. Selection for active and inactive strains. *Anim. Behav.* 14, 444.

DiAntonio, A., Parfitt, K.D., and Schwarz, T.L. (1993). Synaptic transmission persists in *synaptotagmin* mutants of *Drosophila*. *Cell* 73, 1281–1290.

Dotti, C.G., Parton, R.G., and Simons, K. (1991). Polarized sorting of glypiated proteins in hippocampal neurons. *Nature* 349, 158–161.

Elkins, T., Zinn, K., McAllister, L., Hoffmann, F.M., and Goodman, C.S. (1990). Genetic analysis of a *Drosophila* cell adhesion molecule: interaction of fasciclin I and Abelson tyrosine kinase mutations. *Cell* 60, 565–575.

Ellman, G.L., Courtney, K.D., Andress, V., and Featherstone, R.H. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95.

Faivre-Sarrailh, C., Gennarini, G., Goridis, C., and Rougon, G. (1992). F3/F11 cell surface molecule expression in the developing mouse cerebellum is polarized at synaptic sites and within granule cells. *J. Neurosci.* 12, 257–267.

Fischbach, K.-F., and Dittich, A.P.M. (1989). The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure. *Cell Tissue Res.* 258, 441–475.

Fournier, D., Berge, J.-B., Cardoso de Almeida, M.-L., and Bordier, C. (1988). Acetylcholinesterase from *Musca domestica* and *Drosophila melanogaster* brain are linked to membranes by a glycosphospholipid anchor sensitive to an endogenous phospholipase. *J. Neurochem.* 50, 1158–1163.

Fournier, D., Karch, F., Bride, J.-M., Hall, L.M.C., Berge, J.-B., and Spierer, P. (1989). *Drosophila melanogaster* acetylcholinesterase gene structure, evolution, and mutations. *J. Mol. Biol.* 210, 15–22.

Ganetzky, B., and Wu, C.-F. (1982). Indirect suppression involving behavioral mutants with altered nerve excitability in *Drosophila melanogaster*. *Genetics* 100, 597–614.

- Gnagey, A., Forte, M., and Rosenberry, T.L. (1987). Isolation and characterization of acetylcholinesterase from *Drosophila*. *J. Biol. Chem.* 262, 13290–13298.
- Greenspan, R.J., Finn, J.A., and Hall, J.C. (1980). Acetylcholinesterase mutants in *Drosophila* and their effects on the structure and function of the central nervous system. *J. Comp. Neurol.* 189, 741–774.
- Hall, J.C. (1982). Genetics of the nervous system in *Drosophila*. *Q. Rev. Biophys.* 15, 223–479.
- Hall, J.C., Alahiotis, S.N., Strumpf, D.A., and White, K. (1980). Behavioral and biochemical defects in temperature-sensitive acetylcholinesterase mutants of *Drosophila melanogaster*. *Genetics* 96, 939–965.
- Hall, J.C., and Kankel, D.R. (1976). Genetics of acetylcholinesterase in *Drosophila melanogaster*. *Genetics* 83, 517–535.
- Haas, R., Marshall, T.L., and Rosenberry, T.L. (1988). *Drosophila* acetylcholinesterase: demonstration of a glycoinositol phospholipid anchor and an endogenous proteolytic cleavage. *Biochemistry* 27, 6453–6457.
- Hilliker, A.J., Clark, S.H., Chovnick, A., and Gelbart, W.M. (1980). Cytogenetic analysis of the chromosomal region immediately adjacent to the rosy locus in *Drosophila melanogaster*. *Genetics* 95, 95–110.
- Hilliker, A.J., Clark, S.H., Gelbart, W.M., and Chovnick, A. (1981). Cytogenetic analysis of the rosy micro-region, polytene chromosome interval 87D2–4; 87E12-F1, of *D. melanogaster*. *Drosophila Inform. Serv.* 56, 65–72.
- Hoffmann, F., Fournier, D., and Spierer, P. (1992). Minigene rescues acetylcholinesterase lethal mutations in *Drosophila melanogaster*. *J. Mol. Biol.* 223, 17–22.
- Incardona, J.P. (1995). Genetic analysis of glycolipid anchor function using *Drosophila* acetylcholinesterase as a model protein. Ph.D. Thesis. Cleveland, OH: Case Western Reserve University.
- Incardona, J.P., and Rosenberry, T.L. (1996). Construction and characterization of secreted and transmembrane forms of *Drosophila* acetylcholinesterase: an unusually short C-terminal signal for glycoinositol phospholipid anchor addition. *Mol. Biol. Cell* 7, 595–611.
- Karess, R.E. (1986). P element mediated germ line transformation of *Drosophila*. In: *DNA Cloning—A Practical Approach*, vol. III, ed. D.M. Glover, Oxford, United Kingdom: IRL Press, 121–141.
- Kelly, R.B., and Grote, E. (1993). Protein targeting in the neuron. *Annu. Rev. Neurosci.* 16, 95–127.
- King, D.G. (1988). Cellular organization and peritrophic membrane formation in the cardia (proventriculus) of *Drosophila melanogaster*. *J. Morphol.* 196, 253–282.
- Kitamoto, K., Ikeda, K., and Salvaterra, P.M. (1992). Analysis of cis-regulatory elements in the 5' flanking region of the *Drosophila melanogaster* choline acetyltransferase gene. *J. Neurosci.* 12, 1628–1639.
- Landis, D.M.D. (1985). Promises and pitfalls in immunocytochemistry. *Trends Neurosci.* 8, 312–317.
- Lee, J.-D., Kravchenko, V., Kirkland, T.N., Han, J., Mackman, N., Moriarty, A., Leturcq, D., Tobias, P.S., and Ulevitch, R.J. (1993). Glycosyl-phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin. *Proc. Natl. Acad. Sci. USA* 90, 9930–9934.
- Lindsley, D.L., and Zimm, G.G. (1992). *The Genome of Drosophila melanogaster*, San Diego, CA: Academic Press.
- Lisanti, M.P., Caras, I.W., Davitz, M.A., and Rodriguez-Boulán, E. (1989). A glycopospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. *J. Cell. Biol.* 109, 2145–2156.
- Lucchesi, J.C., and Manning, J.E. (1987). Gene dosage compensation in *Drosophila melanogaster*. *Adv. Genet.* 24, 371–429.
- Matter, K., Hunziker, W., and Mellman, I. (1992). Basolateral sorting of LDL receptor in MDCK cells: the cytoplasmic domain contains two tyrosine-dependent targeting determinants. *Cell* 71, 741–753.
- Matter, K., and Mellman, I. (1994). Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr. Opin. Cell Biol.* 6, 545–554.
- Meinertzhagen, I.A., and O'Neil, S.D. (1991). Synaptic organization of columnar elements in the lamina of the wild type in *Drosophila melanogaster*. *J. Comp. Neurol.* 305, 232–263.
- Meinertzhagen, I.A., and Hanson, T.E. (1993). The development of the optic lobe. In: *The Development of Drosophila melanogaster*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Petersen, R.B., and Lindquist, S. (1989). Regulation of HSP70 synthesis by messenger RNA degradation. *Cell Regul.* 1, 135–149.
- Phillis, R.W., Bramlage, A.T., Wotus, C., Whittaker, A., Gramates, L.S., Seppala, D., Farahanchi, F., Caruccio, P., and Murphey, R.K. (1993). Isolation of mutations affecting neural circuitry required for grooming behavior in *Drosophila melanogaster*. *Genetics* 133, 581–592.
- Pirotta, V. (1988). Vectors for P-mediated transformation in *Drosophila*. In: *Vectors—A Survey of Molecular Cloning Vectors and Their Uses*, ed. R. Rodriguez and D. Denhardt, Boston, MA: Butterworths, 437–456.
- Ramón y Cajal, S. (1937). *Recollections of My Life*, translated by E.H. Craigie, Philadelphia, PA: American Philosophical Society.
- Richards, A.G., and Richards, P.A. (1977). The peritrophic membrane of insects. *Annu. Rev. Entomol.* 22, 219–240.
- Robertson, H.M., Preston, C.R., Phillis, R.W., Johnson-Schlitz, D.M., Benz, W.K., and Engels, W.R. (1988). A stable genomic source of P element transposase in *Drosophila melanogaster*. *Genetics* 118, 461–470.
- Robinson, P.J. (1991). Phosphatidylinositol membrane anchors and T-cell activation. *Immunol. Today* 12, 35–41.
- Rosenberry, T.L., and Scoggin, D.M. (1984). Structure of human erythrocyte acetylcholinesterase. *J. Biol. Chem.* 259, 5643–5652.
- Rothberg, K.G., Ying, Y., Kamen, B.A., and Anderson, R.G.W. (1990). Cholesterol controls the clustering of the glycopospholipid-anchored membrane receptor for 5-methyltetrahydrofolate. *J. Cell Biol.* 111, 2931–2938.
- Sargiacomo, M., Sudol, M., Tang, Z.-L., and Lisanti, M.P. (1993). Signal transducing molecules and glycosyl-phosphatidylinositol-linked proteins form a caveolin-rich insoluble complex in MDCK cells. *J. Cell Biol.* 122, 789–807.
- Schubiger, M., Feng, Y., Fambrough, D.M., and Palka, J. (1994). A mutation of the *Drosophila* sodium pump α subunit gene results in bang-sensitive paralysis. *Neuron* 12, 373–381.
- Shiel, M., and Caplan, M. (1995). Developmental regulation of membrane protein sorting in *Drosophila* embryos. *Am. J. Physiol.* 269, C207–C216.
- Srivinas, R.V., Balachandran, N., Alonso-Caplan, F.V., and Compans, R.W. (1986). Expression of herpes simplex virus glycoproteins in polarized epithelial cells. *J. Virol.* 58, 689–693.
- Steller, H., and Pirotta, V. (1986). P transposons controlled by the heat shock promoter. *Mol. Cell. Biol.* 6, 1640–1649.

- Strausfeld, N.J. (1976). *Atlas of an Insect Brain*, Berlin, Germany: Springer-Verlag.
- Strauss, R., and Heisenberg, M. (1993). A higher control center of locomotor behavior in the *Drosophila* brain. *J. Neurosci.* 13, 1852–1861.
- Svebenyi, A. (1969). Cleaning behavior in *Drosophila melanogaster*. *Anim. Behav.* 17, 641–651.
- Tago, H., Kimura, H., and Maeda, T. (1986). Visualization of detailed acetylcholinesterase fiber and neuron staining in rat brain by a sensitive histochemical procedure. *J. Histochem. Cytochem.* 34, 1431–1438.
- Tepass, U., and Hartenstein, V. (1994). The development of cellular junctions in the *Drosophila* embryo. *Dev. Biol.* 161, 563–596.
- Thomas, D.C., Brewer, C.B., and Roth, M.G. (1993). Vesicular stomatitis virus glycoprotein contains a dominant cytoplasmic basolateral sorting signal critically dependent on a tyrosine. *J. Biol. Chem.* 268, 3313–3320.
- Truman, J.W., and Booker, R. (1986). Adult-specific neurons in the nervous system of the moth, *Manduca sexta*: selective chemical ablation using hydroxyurea. *J. Neurobiol.* 17, 613–625.
- Wolfgang, W.J., and Forte, M.A. (1989). Expression of acetylcholinesterase during visual system development in *Drosophila*. *Dev. Biol.* 131, 321–330.
- Zinsmaier, K., Eberle, K.K., Buchner, E., Walter, N., and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of *Drosophila*. *Science* 263, 977–980.