

Evolutionary progression at synaptic connections made by identified homologous neurones

(tetradic and dyadic synapses/photoreceptor synapses/neuronal homology/Diptera/visual synapses)

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ABSTRACT A comparative ultrastructural study of photoreceptor synapses formed upon homologous postsynaptic neurones in insects has been made by using serial-section electron microscopy in representative Diptera from a monophyletic series of 14 families. At all of the synaptic contacts there is a presynaptic dense bar, surmounted in phylogenetically more recent families by a presynaptic platform. Opposite the bar lies a pair of postsynaptic elements that invariably originate one each from two unique monopolar neurones L1 and L2. Both elements contain increasingly elaborate cisternae in more recent flies. Within the phylogenetic series, the postsynaptic ensemble itself changes from the original dyad to a tetradic configuration in more recent Muscomorpha by the addition of two new postsynaptic elements from an amacrine cell. This transition occurs once only in the series, which, gauged by the fossil record, covers divergences from the stem line extending back >200 million years.

During the course of evolution, adaptive modifications of the nervous system must have occurred frequently to account for the many corresponding innovations in the behavioral repertoires of animals. How these evolutionary differences between the brains of related species are expressed at the cellular level remains a mystery. Here we distinguish between two contrasting cellular strategies which could alter brain circuitry during the evolutionary divergence of different phyletic lines: (i) the emergence of new types of neurones, which then become connected so as to modify the circuitry in an existing neuropil, or (ii) alteration in synaptic connectivity among preexisting neurones inherited from a common ancestor. Our comparative study of evolutionary progression in the most peripheral visual neuropil of an insect order, Diptera, supports process (ii) over (i).

Since it is difficult to see any obvious homologies between the neurones of different phyla, the generation of new neurone classes is most likely to have been important at the divergence of the major phyla. Within established phyletic lines, however, this mechanism is contraindicated by examples of conservation of neuronal types, particularly clearly in arthropods, in which many of the larger neurones can be recognized uniquely in both sensory and motor pathways. Individual cases of constancy in neuronal complement and morphology have recently been reported across species and even across orders (1, 2), with a clear possible basis in a common pattern of arthropodan neurogenesis (3). These invariances must in some cases have persisted since the separation from a common ancestor as far back as the Paleozoic era [\approx 300 million years (Myr) B.P.].

Selection within a phylum might instead have favored rearrangement of the synaptic connections extending be-

tween preexisting homologous sets of neurones, but there is no information at this cellular level in the literature. We have therefore undertaken a comparative ultrastructural analysis of an invertebrate sensory synapse, that connecting the photoreceptors of the compound eye with uniquely identifiable postsynaptic neurones in the first optic neuropil, or lamina, of Diptera (the true flies). This synapse is of the multiple-contact type (4) with a group of postsynaptic elements at each presynaptic active zone. A wide variety of such postsynaptic configurations, from monads to pentads (5), has been observed in arthropod sensory systems, even at the same functional site in different species. In the compound eye, photoreceptor synapses provide input to discrete, cylindrical synaptic modules called optic cartridges (see Fig. 1). These have been exhaustively studied in Diptera, but only in *Musca* and related flies from the more recently evolved calyptrate families of the most advanced subgroup Muscomorpha (*Brachycera sensu lato*); along with some families from the older suborder Nematocera, the Muscomorpha are believed to have a monophyletic origin (6-9). Using serial-section electron microscopy, we have discovered that synaptic ultrastructure and, more important, synaptic connectivity have been modified during evolutionary divergence of the Muscomorpha. A preliminary account has appeared (10).

MATERIALS AND METHODS

Most of our insects were caught locally and fixed for electron microscopy within a few hours. After identification to familial level, the head was removed and dissected to allow access to fixative, usually a modified Karnovsky glutaraldehyde/formaldehyde mixture in cacodylate buffer, followed by osmication (11, 12). Material was embedded in epoxy, and ribbons of up to 100 consecutive sections were cut and mounted on carbon-coated Formvar films on slot grids for examination with a Philips 201C electron microscope. Specimen blocks were usually oriented so as to section lamina cartridges transversely near to the medio-frontal border of the eye, close to the equator that separates the dorsal and ventral halves of the eye. Most series of sections covered the middle region in the depth of the lamina cartridge. In addition, series were cut from some species from the extreme proximal edge of the lamina, toward the brain to determine the number of axon profiles passing in a bundle between a single cartridge and the external chiasma.

At least one specimen was examined in detail in this way from each of the following: Muscomorpha: *Musca domestica* (Muscidae), *Melophagus ovinus* (Hippoboscidae), *Lucilia cuprina** (Calliphoridae), *Drosophila melanogaster* (Drosophilidae), *Nemapoda nitidula* (Sepsidae), *Heteropsilopus cingulipes** and *Dolichopus cuprinus* (Dolichopodidae), *Neoratus hercules** (Asilidae), *Bombylius major* (Bombyliidae),

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Abbreviation: Myr, million years.

*Neoxaireta spinigera** and *Microchrysa polita* (Stratiomyidae), *Chrysops* sp. (Tabanidae), *Rhagio mystaceus* (Rhagionidae); Nematocera: *Sylvicola fenestralis* and *Sylvicola dubius** (Anisopodidae), *Bibio* sp. (Bibionidae), *Gymnoplusia* sp.* (Tipulidae); Order Mecoptera: unidentified, probably *Chorista* sp.* (in which * indicates a species from Canberra, Australia; the rest were from eastern Canada).

RESULTS

Known Anatomy of the Cartridge. The neuropil we investigated is highly structured. The optic cartridges in Muscomorpha such as the housefly *Musca* and blowflies *Lucilia* and *Calliphora* are clusters of fixed neuronal composition repeated in a modular array of great regularity. Each cartridge contains a single class of six equivalent photoreceptor terminals arranged in a cylindrical shell and conveying direct input to some of the 12 other uniquely identified neurones (13–17) (Fig. 1). Of the synaptic classes described from the cartridge, the one we have examined in most detail is already thoroughly documented from the recent family Muscidae and repeatedly connects each short photoreceptor axon terminal to small clusters of postsynaptic elements. In the housefly *Musca*, one terminal bears ≈ 200 of these multiple-contact synapses, at each of which four postsynaptic processes are assembled in a regular pattern (refs. 11, 12, and 20; Fig. 1 a–c). The same tetradic pattern is found in the sister group Calliphoridae, blowflies (21).

The identity of the postsynaptic elements is known precisely (11, 12, 22). The two larger median processes at the center of the postsynaptic site (Fig. 1) always come one each from two large monopolar cells, L1 and L2, the trunks of which form the longitudinal axis of the cylindrical cartridge,

in *Musca*. The two smaller, outlying (“polar”) elements at the synapse usually belong to α processes of an amacrine cell (11, 12, 20, 22). To examine the counterpart synaptic contacts in Diptera closer to the ancestral line, evidence for homology across families has first been sought among the participating neurones.

Neuronal Homology Assayed from Golgi Studies and Axon Counts. On the criteria of dendritic branching pattern and axon termination, most and perhaps all of the neurones of the lamina known from the well-studied recent calyptate Muscomorpha (Muscidae, Calliphoridae; refs. 23 and 24) have almost exact structural isomorphs in the advanced acalyptate family Drosophilidae, fruitflies (25), which, although a relatively recent group, probably diverged from a common ancestor shared with the calyptate line >70 Myr ago (26, 27). The lamina neurones are also practically identical in a more divergent acalyptate group, the Syrphidae, hoverflies (23). Golgi preparations have been made from even older families, Dolichopodidae (long-legged flies) and Rhagionidae (snipe flies), and preliminary examination of these again suggests widespread conservation of neuronal form over time (unpublished data).

Our counts from electron micrographs indicate additionally that at least 10 axons run between lamina cartridges and the chiasma in rhagionids and dolichopodids. This compares with the count of an identical number (at least 10 fibers) in the dragonfly *Sympetrum* from the distantly related order Odonata (28) and the claimed count of processes from at least 11 neurones in *Musca* (29), which includes a fiber T1A no longer recognized in more recent accounts. The available evidence thus points strongly to the conclusion that most and perhaps all of the neurones of the muscomorphan lamina have been conserved and that an ontogenetic strategy of

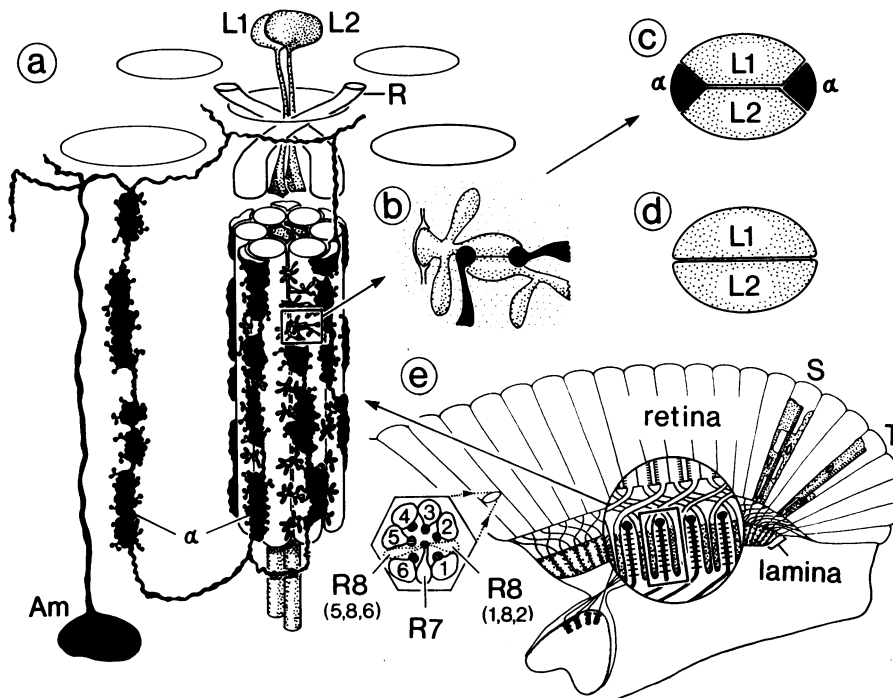


FIG. 1. (a) Much-simplified diagram of a lamina optic cartridge in the housefly, of the relatively recent family Muscidae, Diptera. The basic layout is similar in all higher Diptera (Muscomorpha) examined: six photoreceptor axon terminals (R) converge around two large monopolar neurones (L1 and L2, stippled) that run at the central axis of the cylindrical assembly. Small processes from L1 and L2 participate postsynaptically as a pair at many small tetrad synapses on the surface of each presynaptic R terminal, together with two spiny extensions that usually come from α processes (black) of lamina amacrine neurones (Am). (b) Magnified view of the postsynaptic processes at one synaptic site. (c) Section *en face* through the tetrad of postsynaptic processes at the postsynaptic site. (d) Dyadic arrangement of processes recovered from some older families of Diptera. (e) Position of the cartridge in the eye, the asymmetrical pattern of rhabdomeres in one ommatidium (hexagonal inset), and two different positions of the soma of R8 (dotted outlines) either between R1 and R2 or between R5 and R6; C-type retinæ have only the R(1, 8, 2) pattern, A-type also have the R(5, 8, 6) pattern in the anterior retina, and B-type, likewise, have R(5, 8, 6) in the anterior retina but of the ventral eye only (18). To the right is shown the tiering of the R7 and R8 rhabdomeres, segmented (S) or tandem (T) (19).

developing entirely new morphological categories of neurones has been exercised little, if at all, in the evolutionary development of the optic lobes of Diptera, at least in the most distal visual neuropil.

Changes in the Fine Structure of Synapse-Associated Organelles. Throughout the families of Diptera we sampled, the optic cartridges could be identified as rings of distinctive photoreceptor terminals surrounding the clear axon profiles of two or three large neurones, presumed homologues of the large monopolar cells in *Musca*. In some nematocerans, individual sections of cartridges revealed more than six terminals, but the supernumerary profiles were found to be terminal bifurcations in the few cases traced through serial sections, as would be anticipated from Golgi studies of Bibionidae, March flies (30). The terminals were always identifiable from the presence of occasional pigment granules and from their penetration by branched invaginations from encapsulating epithelial glial cells. These intrusions, called capitate projections (13), were numerous in all forms. They changed appearance within our series (see Fig. 3, feature a) from relatively slender, undifferentiated stalks in Nematocera of the families Tipulidae (crane flies) and Anisopodidae to the form having a prominent apical enlargement with membrane-associated intercellular densities found in higher Muscomorpha [e.g., Muscidae, Hippoboscidae (keds), Drosophilidae].

Synapses from photoreceptors can be recognized readily in all families, although the shape of the synaptic ensemble changes, sculptured around a prominent semilunar ridge extending out from the terminal's surface in lower flies (e.g., Anisopodidae, Rhagionidae, Stratiomyidae, Tabanidae) compared to a smaller terminal elevation already described for the higher calyprates. The presynaptic dense bar present at many insect synapses occurs in all of the families of flies examined, but it too undergoes a transformation in our series. In Nematocera and in Muscomorpha up to the Bombyliidae (beeflies), the bar is simple in form (Fig. 2A and B), but in the Dolichopodidae it becomes a pedestal for a prominent surmounting platform (Fig. 2C), even larger and with a sharper dihedral than in *Musca* and other later groups (Fig. 2D). The characteristic T-shaped appearance in cross section, hitherto thought typical of dipteran synapses in general, is thus a late muscomorph invention. There is an indication of a possible

intermediate condition from faint staining above the pedestal in Bombyliidae.

The photoreceptor synapse in the higher calyprate and acalyprate groups is a tetrad with four processes from different postsynaptic neurones abutting each single presynaptic site (11, 12, 20, 21). Where traced, the two axial monopolar cells of the cartridge, L1 and L2, are the neurones that send branches to occupy the two median locations of the tetrad (Fig. 1c). In recent families, the spine extensions from L1 and L2 contain large flattened subsynaptic cisternae connected to the smooth endoplasmic reticulum (20, 22). These cisternae also undergo a transformation in our series of families. They are not detectable in Nematocera (e.g., Tipulidae), including the family closest to the muscomorph stem line, Anisopodidae (Fig. 2A). Cisternal precursors are recognizable as elongate tubes in some of the most ancient Muscomorpha [e.g., Stratiomyidae (soldier flies), Rhagionidae; arrowheads in Fig. 2B] and first appear in our samples as flattened, especially prominent cisternae in the Dolichopodidae (Fig. 2C).

Alterations in Synaptic Connectivity. The most obviously significant change in the evolution of synaptic morphology lies in modifications we have observed in the synaptic connections. In the Mecoptera, the insect order thought closest to the dipteran ancestral line (6, 7), in Nematocera, and in Muscomorpha in the oldest group of families, Stratiomyidae, Rhagionidae, and Tabanidae, we discovered from serial sections that the synapse is always dyadic, $R \rightarrow L1 + L2$ (Figs. 1d and 2A and B). (This identification assumes that the two clear fiber profiles at the cartridge axis are homologous in all families, as argued above.) Only in Muscomorpha from the Bombyliidae onward is the synapse a tetrad with the addition of two extra polar processes (Figs. 1c and 2C and D). The tetradic structure, hitherto the only form described in the literature, is thus another relatively late muscomorph invention.

It could be argued that the jump from dyad to tetrad synapse is not the result of some infrequent, formidable developmental transition but instead reflects a flexible functional response to some frequent need experienced by the higher flies, perhaps related to some change in habit (for example, the emergence of greater aerial maneuverability might require more sophisticated visual control during flight). Hippoboscidae are recent calyprate flies, distant relatives of

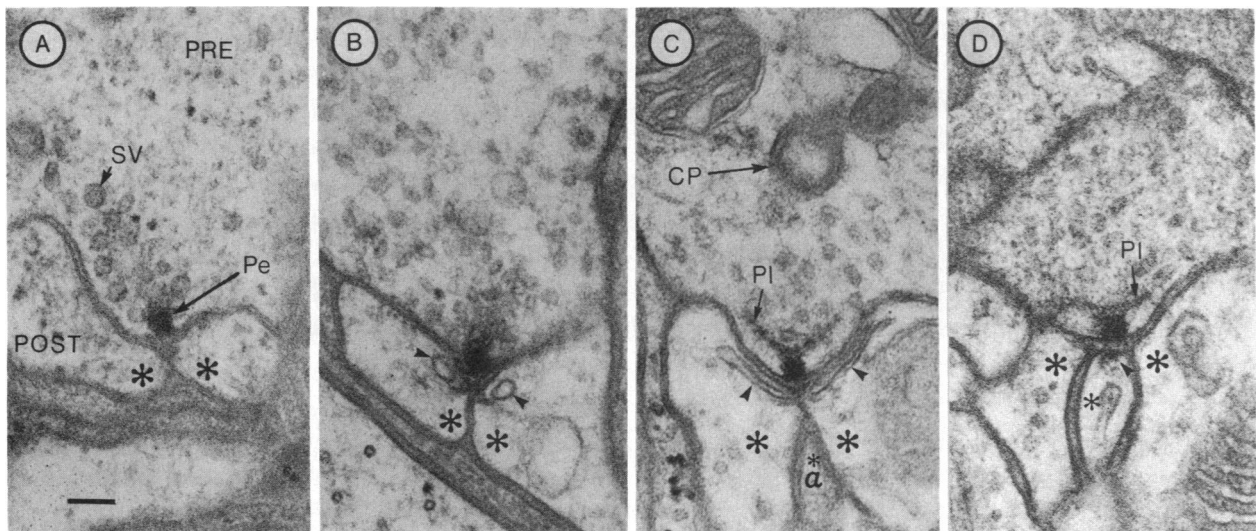


FIG. 2. Photoreceptor synapses from Diptera, in ascending phylogenetic order from Anisopodidae (A), Stratiomyidae (B), Dolichopodidae (C), and Hippoboscidae (D). Two postsynaptic processes (*) of dyads or three of tetrads are visible in each of the synaptic contacts in single transverse sections. Pe, pedestal; PI, presynaptic platform; CP, capitate projection; SV, synaptic vesicles; PRE and POST, the pre- and postsynaptic elements at the synapse; α , α element. (Bar in A, 0.1 μ m, applies to A–D.) ($\times 59,000$.)

the Muscidae, which have specialized as vertebrate parasites, providing a test case of retrogression from the original free-flying habit. The most extreme obligate ectoparasitic form, the sheep ked *Melophagus ovinus*, lives permanently as a sluggish, wingless adult deep in the fleece of sheep in conditions that must approximate scotopic. Its eye is much reduced in facet number, but the details of its photoreceptor terminals resemble those of an advanced muscid or calliphorid, including the presence of well-developed capitate projections and, at the synapse itself, a presynaptic platform and clear tetradic architecture. Densities occurring in some postsynaptic processes (arrowhead, Fig. 2D) may be membranous cisternae or their electron-dense "whiskers" as described in *Musca* (11, 20).

DISCUSSION

A monophyletic origin is a necessary prerequisite when examining progressive evolutionary changes in any structure. Although some families have been insufficiently investigated, there is general agreement in all recent taxonomic reviews that the Muscomorpha as a group have a monophyletic origin (6-9) from nematoceran ancestry probably at least as far back as the Jurassic period. Some groups of less interest here may be unnatural paraphyletic assemblages (6), and there is disagreement among specialists about the rank appropriate for certain taxa and about details of evolutionary divergences. Nevertheless, a broad enough consensus exists about the subgroupings of most of the constituent families and about the overall sequence of evolutionary divergence (6-9, 31) to suffice for interpretation of the present study. The relict nematoceran family Anisopodidae, first known as fossils from the upper Triassic, may be the group closest to the muscomorphan stem line (refs. 9 and 31; Fig. 2A).

Neurological evidence corroborating the monophyly of the muscomorphan line comes from the peculiar asymmetrical pattern of rhabdomeres in the ommatidium (32). The pattern needs to be closely regulated to maximize its optical advantage, as described for *Musca* (33), and has been found universally in the Muscomorpha examined so far (18, 19, 32). This further example of cellular evolutionary conservatism in fact provides the only known taxonomically useful feature open to external observation that uniquely characterizes adult Muscomorpha. The Nematocera, even those closest to the muscomorphan stem line, lack this pattern: Anisopodidae (unpublished data); Bibionidae (30). Golgi studies, including the early survey of the deeper visual neuropils of Tabanidae (34), demonstrate an obvious isomorphism between neurones of groups which diverged hundreds of millions of years ago, thus supporting a monophyletic origin for the Muscomorpha as a whole. Along with clear morphological differences from neurones in the laminae of other insect orders (35), this provides persuasive evidence that the neurones of the lamina can be homologized across different families of Diptera. For some neurones of the visual system, isomorphism extends even more widely among arthropods. A striking example is lamina visual interneurone T1, which has a distinctive and practically invariant branching pattern and location in crustaceans and in insects (25). For all of these cases, morphological evidence in favor of their homology in different families needs to be supplemented by evidence of a common ontogenetic origin of the cells.

A summary of findings on retinal and synaptic morphology in Fig. 3 attempts to relate these to an approximate time scale of Dipteran evolution and reconstruct a dendrogram based upon the points of implied divergence. Two features are included from studies by Wada (18, 19), who described interfamilial variations in the position of photoreceptor R8 in the ommatidium (row f, and Fig. 1e) and in the pattern of

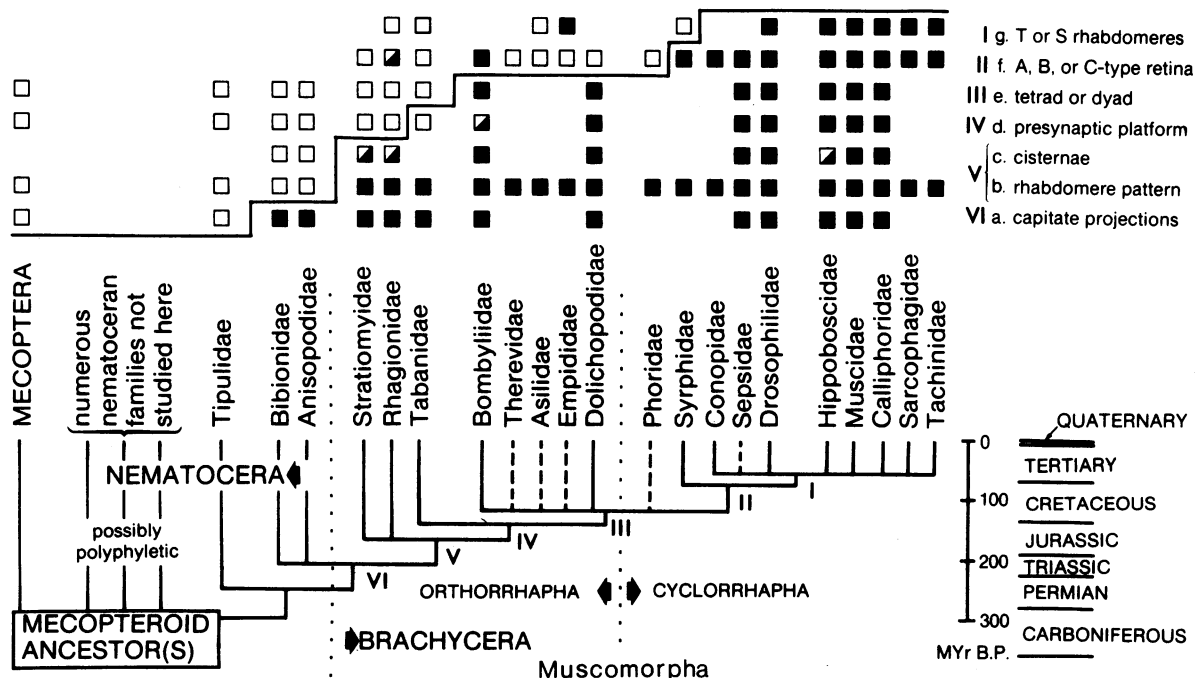


FIG. 3. Phylogenetic relationships of some modern families of Diptera. The geological time scale at the right is consistent with recent estimates based on protein sequencing (26, 27). The checklist at the top identifies the possession by the family of certain advanced characters (■) of the photoreceptor synapse or of eye design. □, Original, ancestral condition; ▣, intermediate form. The attributes, arranged in ascending order of implied antiquity, indicate the presence of glial capitate projections (row a) (13); an asymmetrical pattern of photoreceptive rhabdomeres (row b) (18, 19); cisternae in the two central postsynaptic processes (row c); a distinct platform atop the presynaptic pedestal (row d); tetrad photoreceptor synapses (row e) (□, dyads); C- (■), B- (▣), or A-type (□) retina (row f) (18); T- (■) or S- (□) arrangement of R7 and R8 rhabdomeres (row g) (Fig. 1e) (19). Features in rows a-g identify six steps, I-VI; these have been used to generate the actual dendrogram below, which agrees substantially with other taxonomic schemata (6-9). Interrupted lines are drawn if the diagnostic feature of a step has not directly been observed in a particular family, the phylogenetic position of which is given instead by its other features.

tiering of rhabdomeres of R7 and R8 at the dorsal margin of the eye (row g). The several structural alterations observed by him and us appear not to be obligatorily linked to one another, since all but two first make an appearance in different families, and persist thereafter in all of the subordinate branches of the dendrogram that have been examined.

Although based on an incomplete analysis of just a few neural features in a small number of families, our implied steps I–VI (Fig. 3) are in surprisingly close agreement with recent, independently derived dendrograms based on multiple taxonomic characters. In particular, Steyskal's (8) steps S1–S10 and Hackman and Väisänen's (9) steps HV1–HV6 correspond as follows: our step I is equivalent to S3, and step II is equivalent to S4, except that we exclude the Phoridae (coffin-flies); step III disagrees with the tentative radiation S5 but agrees with HV2; step IV has no exact counterpart; step V concurs with S6 and HV3; and step VI concurs with S7 and HV4. A minor resulting unorthodoxy in our scheme is that the phorids are displaced further from the acalyptates than either authority (8, 9) supposes, but most authors acknowledge that this family's affinities are particularly obscure. Step IV implies rearrangement in the sequence of the stratiomyid-rhagionid-tabanid divergence, isolating the tabanids more toward the bombyliids, compared with ref. 8. The two characters out of place among features a and b of Fig. 3, in bombyliids and empidids, concern the retinal features taken from Wada (18, 19). The concordance of our scheme and others' (8, 9) suggests that the dendrogram of Fig. 3 may accurately rank the several neural alterations in the order of their acquisition in phylogeny. All of the synapse-associated modifications in Fig. 3 reflect a phylogenetic trend toward an increase in size or complexity of the individual organelle over time, while the overall absolute size of the postsynaptic ensemble has remained approximately constant, despite variations in cell size.

The synapse-associated changes reported here are presumed to have operational significance. There is little direct indication of what functional change attends the switch from dyad to tetrad, but a candidate possibility is an increase in visual time resolution. In calliphorids, the same amacrine processes that are postsynaptic to receptor terminals at the tetrad are also locally presynaptic to them at numerous large reciprocal synapses, suggestive of a local feedback loop (17). This class of amacrine synapse is absent in *Musca*, however, and appears to have been secondarily lost. Terminal responses recorded during visual activity in calliphorids exhibit a rapid transient negative feedback with spatial characteristics that have been interpreted as indicating amacrine involvement (17). The effect should be to curtail rapidly transmission through the photoreceptor output synapse in the cartridge, increasing the frequency response for transient stimuli. This could have had survival value during early evolution, for, unlike most ancient dipteran groups, some species in the more recent families habitually make frequent fast angular corrections during flight. While tracking conspecifics visually, for example, overall reaction time can be as short as 12 msec, emphasizing the need for enhanced temporal resolution (36, 37).

Our results to date thus support the idea of neuronal conservation (1–3). Homologous neurones may persist identifiably for long periods of time even by geological standards, for L1 and L2 in Diptera at least since the original Carboniferous mecopteroid ancestors >300 Myr ago. In contrast, the implication from our comparative study is that the connections that these identifiable neurones make have been somewhat more modifiable when considered on the geological time scale. Even these changes were very infrequent in the cases

we studied in the visual system. Thus, in the middle term, the potential for alteration of synaptic connectivity may constitute not only the main avenue but also the main restriction upon possible evolutionary changes in nervous organization. If this finding on Diptera proves to be a general rule for other nervous systems, then the quest to explain evolutionary divergences can be seen to have converged with a basic aim of developmental neuroscience—that of defining the mechanisms by which particular synaptic connections are formed, respecified, or abandoned.

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