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Modification of dispersal patterns of branched photoreceptor axons and the evolution of neural superposition

R. R. Melzer*, T. Zimmermann and U. Smola

Zoologisches Institut, Luisenstr. 14, D-80333 München (Germany), Fax +49 89 5902 450,
e-mail: melzer@zi.biologie.uni-muenchen.de

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Abstract. Using 3D reconstructions of serial ultrathin sections and extended-focus pictures of Golgi-impregnated and cobalt-stained visual fibres, we studied the branched short photoreceptor axons found in ancestral dipterans and in the scorpionfly *Panorpa*. In the 'phantom' midge *Chaoborus* (Nematocera), each cartridge of the lamina neuropil is innervated by collaterals of 24 photoreceptor axons from 18 neighbouring and next-but-one ommatidia, in a regular pattern of asymmetrical neural pooling. Comparison of axon morphologies in different groups (Tipulidae, Chironomidae, Culicidae) indicates that this pattern must represent an ancestral condition of the dipteran visual system and is thus a precursor of neural superposition found in flies (Brachycera) in which only one set of photoreceptors R1–R6 converges onto each cartridge instead of the four sets found in *Chaoborus*. It can be concluded that specific axonal input channels from the large array of innervating photoreceptors found in midges have been retained during the evolution of neural superposition.

Key words. Retinula axons; collateral pathways; synaptic connectivity; neural superposition; evolution; Nematocera (Diptera, Insecta).

The compound eyes and optic lobes of insects are composed of highly conserved sensory and neuronal elements [1]. These form arrays of repeated subunits, referred to as ommatidia at the level of the compound eyes, and as cartridges or columns at the level of the sensory neuropils. Generally, each ommatidium innervates a single cartridge representing the same point in space as the ommatidium, and thus a highly specific retinotopic map of the visual environment is established. This is maintained between the first-, second- and third-order neuropils by sets of specific neurons found in each cartridge [2, 3].

Changes in synaptic connectivity between these elements rather than the evolution of new neurons have been shown to bring about the wide range of sensory capacities of this system [4]. Neural superposition, as found in flies like *Drosophila* and *Musca*, constitutes one such mechanism. Here the short photoreceptor axons, that in other insects normally innervate a single neuronal unit in the lamina neuropil [3], have changed their target areas so that six photoreceptors, distributed among neighbouring ommatidia and looking at the same point in space, provide input to one channel [5, 6]. This system combines a high signal-to-noise ratio with high spatial resolution [6, 7].

Neural superposition is found in the most primitive brachyceran groups [8–10], so that the key to understanding its evolution must be sought in the flies' closest relatives, the nematocerans. For this group, two types of visual processing have been suggested: A special type of neural superposition in male marchflies (Bibionidae) [11, 12], and asymmetrical neural pooling in craneflies (Tipulidae) [13]. In addition, branched photoreceptor axons with collaterals projecting tangentially across the neuropil have been found as putative neuroanatomical correlates of these mechanisms [8, 9, 11, 12, 14].

In this paper we compare the shape and arrangement of axons in different nematocerans and in one of their closest relatives, the ancestral scorpionfly *Panorpa*, and combine these findings with the synaptic connectivity of axon collaterals in the 'phantom' midge, *Chaoborus*, into a neuronal pattern that can be considered as the precursor of the types of neural superposition described in more recent dipterans. In order to determine the shape of the short retinula axons in the dipteran basic plan, we have chosen representatives of Tipuliformia and Culiciformia that are generally seen as closest to the ancestral dipteran stock [15]. In addition, the mecopteran *Panorpa*, belonging to the the sister group of the Diptera [16] is an appropriate model to determine whether branched photoreceptor axons are a derived character of dipterans or whether they evolved earlier in the phylogeny of Holometabola.

Materials and methods

Electron microscopy and 3D reconstruction of ultrathin sections

Light-adapted females of *Chaoborus crystallinus* (De Geer, 1776) (Chaoboridae, Nematocera) were fixed simultaneously in glutaraldehyde and osmium tetroxide in 0.1 M cacodylate buffer [17] and embedded in epoxy

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* Corresponding author.

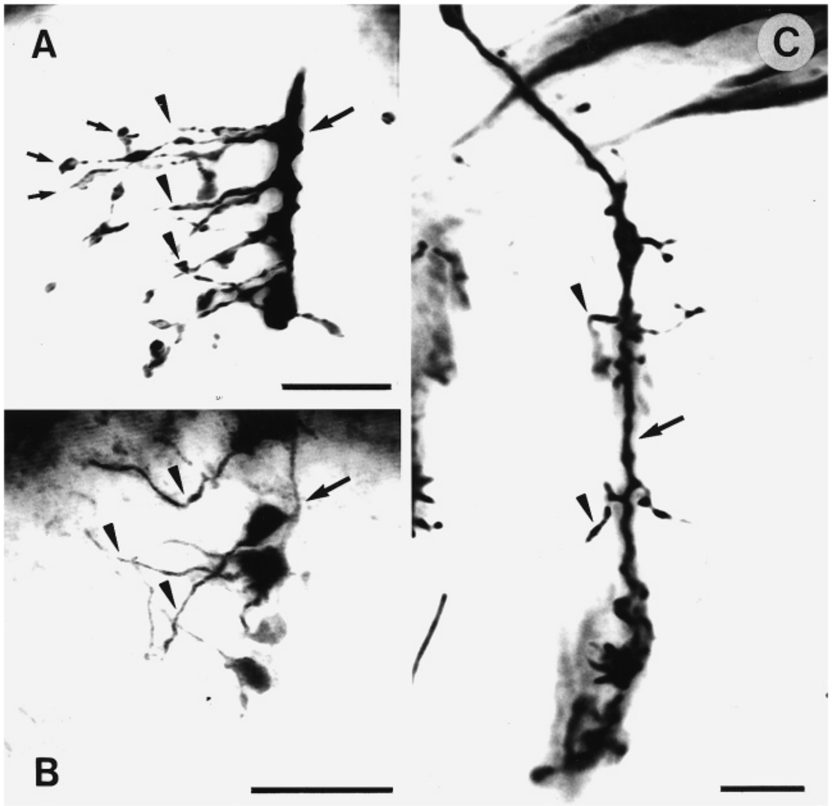

Figure 1. Extended-focus videomicrographs of short photoreceptor axons (R1–R6) in *Tipula rufina* (*A*), *Chaoborus crystallinus* (*B*) and *Panorpa vulgaris* (*C*). (*A*, *C*) Golgi impregnation, (*B*) cobalt fill. Note length and arrangement of axon collaterals (arrowheads) projecting from the main axons (large arrows) to optical cartridges in the neighbourhood; small arrows show secondary branches of the most distal collateral in *Tipula*. Bar 10 µm.

resin. After approaching the target area by 1 µm cross-sections of the region near the eye's equator, series of up to 300 ultrathin sections of 100 nm thickness comprising the distal cell-body rind and the lamina neuropil were cut with a diamond knife on a RMC 7000 ultramicrotome. The sections were stained with uranyl actetate and lead citrate in a LKB 2168 Ultrostainer and observed in a Philips CM10 electron microscope at 80 kV. The most complete series of cross-sections through the neuropil was photographed at 2950×, with the same visual column always being placed in the centre of the micrograph so that the morphology and synaptology of elements of visual columns and their interaction with neighbouring cartridges could be analysed. Relevant structures were marked and traced through the series of photographs. After scanning and transferring to an IBM RS 6000 workstation, the images were aligned using programs written for CreaSo's Interactive Data Language (IDL) software package. With the same software, structures of interest were isolated and rendered in three dimensions.

Light microscopy and extended-focus pictures

Heads of adults of the scorpionfly *Panorpa vulgaris* (Panorpidae, Mecoptera) and the nematocerans *Tipula rufina* (Tipulidae), *Chironomus nubilus* (Chironomidae), *Culiseta annulata* (Culicidae) and *C. crystallinus* were submitted to two cycles of the Golgi-Colonnier method [18, 19]. In addition, cobalt fills of retinula axons of *C. crystallinus* were made according to standard procedures [reviewed in 20, 21]. Cobalt crystals were inserted via lesions in the cornea made with insect needles '000' or a tungsten wire previously etched in 1 N NaOH at 12 V. After embedding, 8–20 µm sections were made.

Extended-focus images of stained retinula axons were created with a CCD-camera mounted on a Zeiss Axioplan microscope connected to a PowerMac 7500. Using the program NIH Image 1.60 β, an extended depth of field was achieved by combining images taken at different focal planes into a single picture containing the elements of all 'optical sections' [22].

Results

In all the species examined, using Golgi- and cobalt-staining we observed branched photoreceptor axons. The axons of the outer retinula cells R1–R6 had three to five groups of collaterals originating at different neuropil depths (fig. 1). Unlike those found using cobalt-staining of axons in larval *Chaoborus* [23], the collaterals were long in all the adults studied. Generally, they were bifurcated and showed an initial varicosity at or

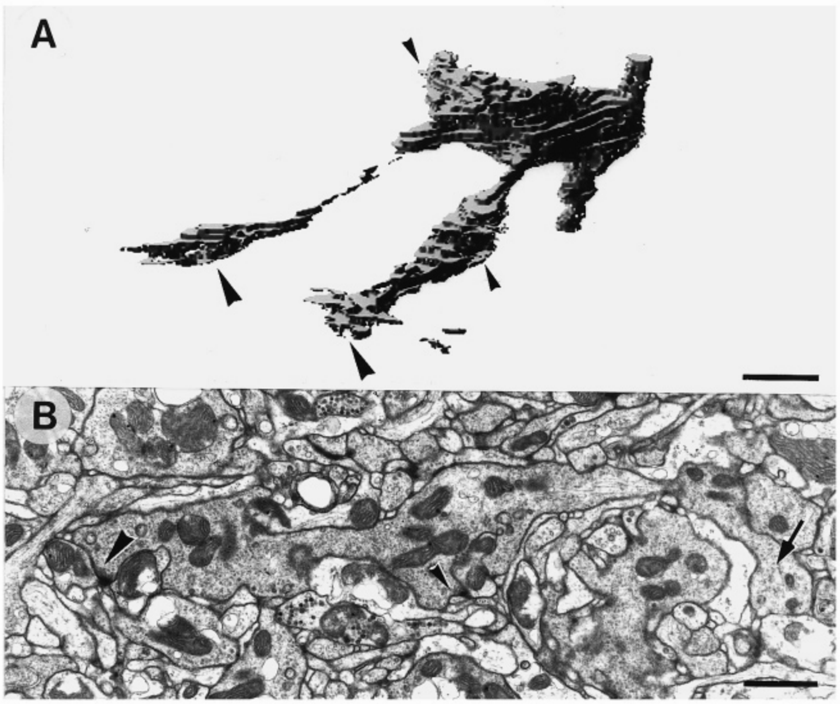

Figure 2. (*A*) 3D reconstruction of serial ultrathin sections of a bifurcated collateral in *Chaoborus*; small arrowheads indicate presynaptic sites in a neighbouring optical cartridge, large arrowheads synapses onto a next-but-one cartridge. Bar 2 μm. (*B*) Longitudinal section of a collateral with synapses in a neighbouring (small arrowhead) and a next-but-one optical cartridge (large arrowhead); arrow main axon. Bar 1 μm.

near the main axon. Originating from the varicosity were fine secondary branches that projected tangentially across the neuropil. The most distal and longest collaterals in *Tipula* had three branches rather than two (fig. 1A). The collaterals of a single short axon had the same target areas in their respective depth of the neuropil, while those of the six short axons of each ommatidium diverged from each other. This implies convergence of the collaterals of photoreceptor axons from neighbouring ommatidia. While the shape and branching pattern of the short axons in the examined nematocerans was highly uniform, we observed axons of diverse shape in *Panorpa*. In addition to those already described [24], we found axons with a dominant main branch and collaterals lacking the brush-like orientation seen in the nematocerans (fig. 1C; see also [24]).

In adult *Chaoborus*, tracings of profiles through serial ultrathin sections spanning the whole depth of the lamina, and their 3D reconstructions, showed that the collaterals of photoreceptor terminals ran back and forth between the optical cartridges, exhibiting a highly regular arrangement and synaptic pattern, one that is identical for all collaterals of one retinula axon. Originating from the short axon's main branches the collaterals projected away from their parent cartridge and contacted other cartridges in the neighbourhood. As shown by the distribution of their synaptic contacts (fig. 2), each bifurcated collateral not only innervates a single cartridge, but is instead presynaptic to two cartridges directly neighbouring the primary one as well as two next-but-one cartridges, i.e. the short axons of a single ommatidium innervate all the surrounding 18 cartridges, while there is no synaptic input to the underlying cartridge (fig. 3A). Conversely, each cartridge receives input from 24 photoreceptors, 12 of the first and 12 of the second ring of ommatidia. The 12 inputs of the first ring were composed of two adjacent retinula cells from each of the six ommatidia found here, while those of the second ring were formed by one retinula cell from each of the 12 next-but-one ommatidia (fig. 3B). Via collaterals, four cells of each of the six photoreceptors R1–R6, originating from four ommatidia directly bordering one another, converged upon a single optical cartridge, instead of each of R1–R6, the pattern found in brachycerans [6]. Two retinula cells were from the first and two from the second ring of ommatidia. Each subadjacent ommatidium contributed to one group of four, and each adjacent ommatidium to two of them.

As postsynaptic elements in each cartridge we identified two large monopolar neurons forming dyad synapses with the axon collaterals. Therefore these neurons must represent L1 and L2 [see also 9, 14 and 25]. As found in dipterans with neural superposition, the axon bundles of the nematocerans studied also performed a 180° rotation distal to the lamina neuropil, leading to the projection pattern shown in figure 3C for *Chaoborus*.

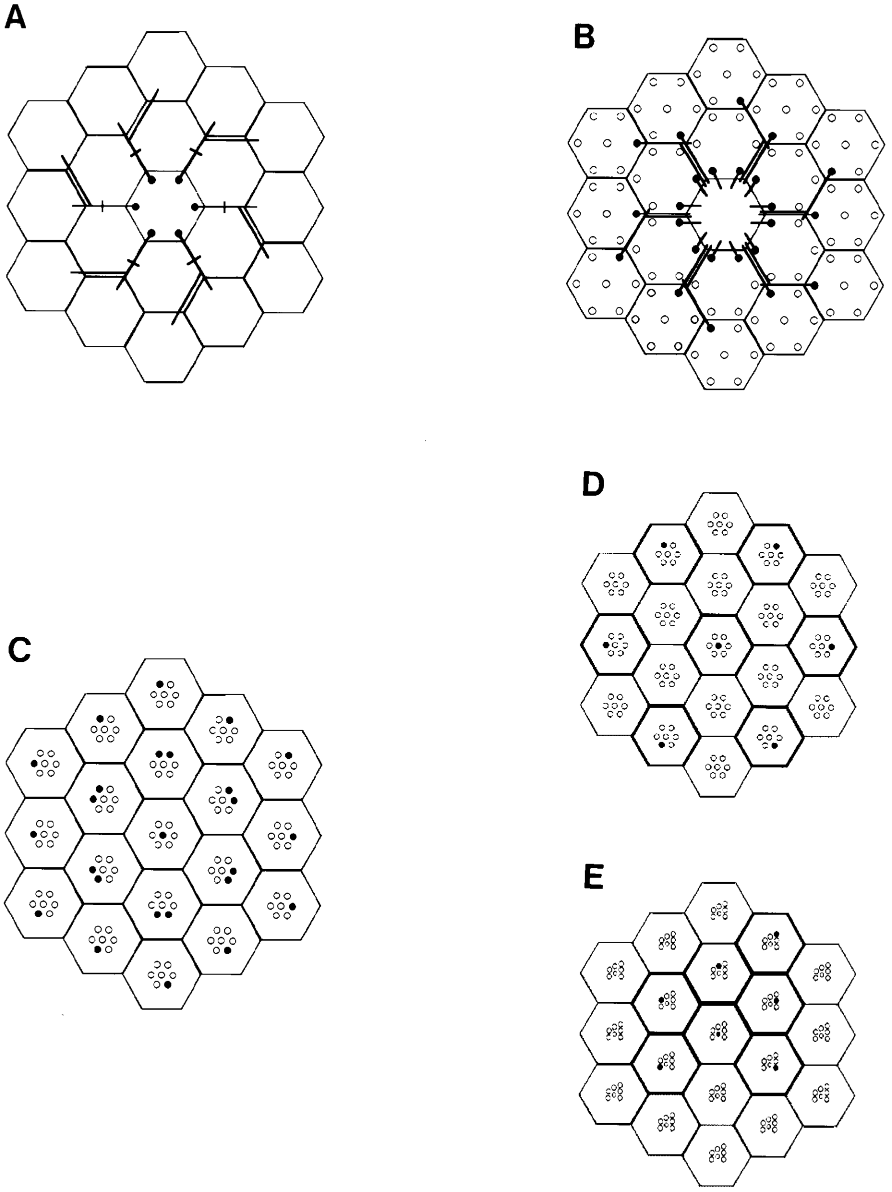

Figure 3. Wiring of short retinula axons in *Chaoborus* (*A*–*C*) and other dipterans (*D*, *E*). (*A*) Synaptic connections of axon collaterals diverging from their primary cartridge to the neighbourhood. (*B*) Collaterals from the 18 neighbouring and next-but-one cartridges converging onto the central cartridge. (*C*) Array of retinula cells that innervate the central optical cartridge at the ommatidial level before 180° rotation of the axon bundles distal to the lamina. (*D*, *E*) Two types of neural superposition described for Diptera: (*D*) dorsal eye of male *Bibio* (after [11, 12]), (*E*) the eye of the fly (after [6]). As in (*C*), dark circles indicate photoreceptors that project to the central optical cartridge.

Discussion

Given the generally accepted outlines of dipteran phylogeny [15], the presence of collaterals in several major nematoceran groups leads to the conclusion that convergence of visual information from different ommatidia onto a single input channel via regularly arranged collaterals is an ancestral feature of the dipteran visual system, and hence must represent a precursor of classical neural superposition. During evolution, spreading collaterals like those found in the sister group of the dipterans, the scorpionflies, appear to have invaded the cartridges neighbouring the one underlying their own ommatidium of origin, forming the divergent inputs to nematoceran cartridges. The different *Gestalten* of short retinula axons described for *Panorpa* [see also 24] indicate that there might be other and possibly less regular modes of wiring to neighbouring cartridges, ancestral to the pattern of nematocerans.

The collateral arrangement and synaptic pattern found in Nematocera correspond well to the asymmetrical neural pooling of visual information that has been inferred from optical measurements in a cranefly, a mode that provides a maximum of light sensitivity in the dark-adapted state [13]. While the bifurcated collaterals in *Chaoborus* innervate two surrounding rings of cartridges, the trifurcated and very long distal collaterals of *Tipula* are consistent with the measurements of the visual fields of ommatidia in this group, showing overlap between ommatidia as much as three rows apart [13].

As shown in figure 3B, terminals converge from neighbouring ommatidia in a way that perfectly correlates with the particular cell of origin, R1–R6, and its spatial arrangement within the ommatidium. Both types of neural superposition described for dipterans, including the trapezoidal pattern of the fly eye, can be directly derived from the wider patches of cartridges innovated by large nematoceran ommatidia by the selective reduction of inputs from photoreceptors from adjacent or subadjacent ommatidia (fig. 3C–E). One can therefore conclude that neural superposition was brought about by retention of specific input channels, e.g. those providing the highest spatial resolution, rather than by directed growth of inputs to specific cartridge targets.

Optimizing spatial resolution requires a precise focussing of the rhabdomeres on a single point in space, which may explain why only one retinula cell of each of R1–R6 innervates an optical cartridge in bibionids and in brachycerans instead of the four cells in *Chaoborus*. The modes of neural superposition described so far thus reflect different possibilities of selecting one set of R1–R6 out of the larger arrays that innervate a single cartridge in ancient midges. In the dorsal eye of *Bibio* males [11, 12], a type using only next-but-one retinula cells has evolved. The trapezoidal rhabdom pattern in brachycerans that appears to combine retinula cells of five adjacent and one subadjacent ommatidia can be seen as another way of selecting six out of 24 terminal inputs. Yet another type of reduction of input of the four R1–R6 sets of chaoborids to a single one is that of the female eye of the bibionid *Dilophus* [12], where retinula cells of four adjacent and two subadjacent ommatidia converge. According to Zeil [12], these types represent different solutions to the problem of minimizing both the interrhabdomere and interommatidial angles of directly neighbouring ommatidia that generate asymmetrical rhabdom patterns. As shown here, all these types may have had a common origin in the large arrays of several sets of R1–R6 found in ancestral nematocerans.

Findings on the optics of a nematoceran compound eye suggest that ancestral dipterans evolved neural pooling in an effort to increase light sensitivity, and subsequently modified the pooling fields in a trade-off between sensitivity and resolution, culminating in the types of neural superposition found in flies and bibionids [13]. This model is well supported by studies on the evolution of the dipteran rhabdom [26], the morphological changes of the rhabdom during light and dark adaptation [27], and the morphology of lamina neurons [8, 9, 11, 12, 14, 25]. Our findings show that these evolutionary changes are accomplished at the level of first-order neurons by means of the collateral pathways of their receptor axon inputs.

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