The Journal of Experimental Biology 199, 2395–2407 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
JEB0484

NEW FEATURES OF THE LOCUST OPTIC LOBE: EVIDENCE OF A ROLE FOR NITRIC OXIDE IN INSECT VISION

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Accepted 26 July 1996

Summary

The enzyme nitric oxide synthase can be localised by NADPH-diaphorase histochemistry. Here we have applied this technique to the optic lobe of the locust *Schistocerca gregaria* and revealed new features of the insect visual system. Extensive but locally intense staining is associated with identified tracts, distinct neuropiles and cell body groups, and a detailed analysis of stained elements is provided here. The most striking staining occurs in the anterior lobe of the lobula complex and its connection with the medulla by means of the dorsal uncrossed bundle. Eleven groups of cell bodies are identified and their contribution to fibre tracts and neuropile areas is described. Diaphorase-positive fibre tracts pass between all major subdivisions of the optic lobe, but there are no conspicuous fibre connections from the optic lobe to the brain. The widespread distribution of NADPH-diaphorase staining in the optic lobe suggests that nitric oxide is likely to play an important role in information processing in insect vision.

Key words: nitric oxide, nitric oxide synthase, insect, optic lobe, vision, NADPH-diaphorase, locust, *Schistocerca gregaria*.

Introduction

The gas nitric oxide (NO) was first identified as a neuronal signalling molecule in the mammalian central nervous system (Garthwaite *et al.* 1988). Neurones that produce NO contain a constitutive Ca^{2+}/calmodulin-activated NO synthase (NOS) (Bredt and Snyder, 1990; Bredt *et al.* 1991*a*,*b*) and NO is thought to act as an intercellular signalling molecule by diffusing from sites of synthesis and interacting with receptor proteins in adjacent cells. The principal target of NO in the nervous system is the soluble form of the enzyme guanylyl cyclase (Garthwaite, 1991).

NOS-containing neurones are present throughout the mammalian central nervous system (CNS) but are particularly abundant in the cerebellum, the dentate gyrus of the hippocampus and the olfactory bulb (Bredt *et al.* 1991*a*). The NO–cyclic GMP signalling pathway is, however, not unique to mammals but also exists in the simpler nervous systems of the two major invertebrate groups, insects (Elphick *et al.* 1993; Müller and Buchner, 1993) and molluscs (Gelperin, 1994). In insects, the brain of the desert locust *Schistocerca gregaria* contains the two key component enzymes of the NO–cyclic GMP pathway, namely a Ca^{2+}/calmodulin-activated NOS and an NO-activated guanylyl cyclase. Moreover, the locust NOS, like its mammalian neuronal counterpart, is NADPH-requiring, produces NO and citrulline from L-arginine and is inhibited by N^{ω}-nitro- and N^{ω}-methyl- analogues of L-arginine (Elphick *et al.* 1993, 1995). Recently, a gene encoding a protein with 43 % amino acid identity to rat neuronal NOS was reported in *Drosophila melanogaster* (Regulski and Tully, 1995), providing further evidence that NO is a signalling molecule in the insect CNS.

The NADPH-diaphorase histochemical method is known specifically to stain NOS-containing neurones in aldehyde-fixed mammalian CNS (Hope *et al.* 1991; Dawson *et al.* 1991), where selectivity appears to be conferred by an unusual ability of NOS to retain NADPH-diaphorase activity after aldehyde fixation (Matsumoto *et al.* 1993). In the locust, the NADPH-diaphorase method reveals intense staining in the olfactory lobes of the brain with rather less intense staining in other central brain structures. Measurement of NOS activity in different brain regions, using the arginine-to-citrulline assay, shows a correspondence between high levels of Ca^{2+}/calmodulin-dependent NOS activity and high levels of diaphorase staining (Elphick *et al.* 1995). Thus, in the insect CNS, as in mammals, NADPH-diaphorase can be used to indicate the anatomical localisation of neuronal NOS.

Here we describe the anatomical distribution of NADPH-diaphorase staining in the visual processing centres of the locust brain, the optic lobes. A highly repeatable pattern of staining in a diverse population of neurones is revealed, indicating that NO is likely to play an important role in visual

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processing and visually guided behaviour in insects. In its entirety, the distribution of NADPH-diaphorase staining does not correspond with the overall picture revealed by other histological procedures. This suggests that NOS is not associated or co-localised in any simple way with any other single chemical signalling pathway involved in vision.

Materials and methods

Animals and chemicals

The locusts (*Schistocerca gregaria* Forskål) used in this study were reared in our laboratory cultures or purchased from Blades Biological (Edenbridge, Kent, UK). Animals used were mature adults (male and female). Chemicals used were purchased from Sigma unless otherwise stated.

Histochemical techniques

For NADPH-diaphorase histochemistry, the exoskeleton was dissected from the anterior face of the locust head to expose the brain and optic lobes. Heads were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.3) at 4 °C for 4 h and cryoprotected in 10% sucrose/PBS overnight at 4 °C. The brain and optic lobes were removed and embedded in Tissue-Tek O.C.T. Compound (Miles Inc. USA). Serial 30 µm frozen sections were cut using a Leica CM 3000 cryostat and collected on chrome-alum/gelatin-coated slides. Slides were washed in 50 mmol l^{-1} Tris–HCl (pH 7.5) and then incubated in 1 mmol l^{-1} NADPH/0.25 mmol l^{-1} Nitro Blue Tetrazolium in Tris-HCl at room temperature (in the dark) for about 1 h. After washing in water, sections were mounted with an aqueous mounting medium (Immun-mount, Shandon). Golgi impregnation was carried out using Valverde's (1970) modification of Cajal's rapid Golgi method. Brains were embedded in an epoxy resin and sections cut at 40 µm.

Results

Orientation, structure and terminology

During embryogenesis, the head rotates backwards by about 90° with respect to the body axis such that the brain is orientated at right angles to the rest of the neuraxis. Thus, in the locust, the front of the brain is topologically ventral, the back of the brain is topologically dorsal and the top of the brain is topologically anterior. Accordingly, all orientations used in this paper refer to the embryonic neuraxis (Boyan *et al.* 1993). This is a potential source of confusion that is clarified in Fig. 1, which places the optic lobes in the context of the brain as a whole.

The optic lobes are divided into three retinotopic neuropilar regions arranged along the axis of the visual processing pathway from distal to proximal and named respectively the lamina (La), medulla (Me) and lobula (Lo) (Fig. 2A). These neuropiles are connected by the first optic chiasma (OCh.I) which joins the lamina with the medulla and the second (OCh.II) which joins the medulla with the lobula (Gouranton, 1964). Anteriorly in both the lamina and the medulla there are

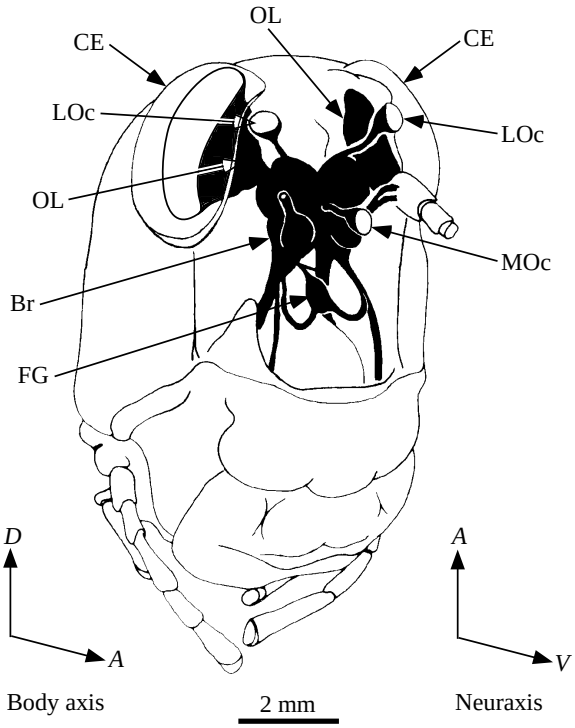

Fig. 1. Diagram of the brain showing its position in the head and its orientation in relation to the body axis and neuraxis. Note the position of the optic lobes (OL) between the compound eyes (CE) and the brain (Br). Other structures shown are the median (MOc) and lateral (LOc) ocelli and the frontal ganglion (FG). Reference axes are dorsal (*D*), ventral (*V*) and anterior (*A*). Modified from Williams (1975).

discrete anatomical regions known as anterior rims. While their function in the locust visual system is not known, in crickets the anterior rims of both the lamina and the medulla (ARLa and ARMe) have been shown to be involved in the detection of polarized light (Labhardt and Petzold, 1993). Ventral to the main medulla there is a small, non-retinotopic satellite neuropile known as the accessory medulla or AccMe (Fig. 2A) (Pflugfelder, 1936/37).

The lobula is associated with a number of neuropilar compartments (Gouranton, 1964) that are not retinotopic and that probably derive from outgrowths of the midbrain, partly enveloping the true lobula. While the exact origin of these structures has not been established, for consistency we have renamed them with respect to the embryonic axis as follows: anterior lobe (AL), dorsal lobe (DL), dorso-medial lobe (DML) and ventral lobe (VL) (Fig. 2B). These lobula-associated, non-retinotopic neuropiles together with the retinotopic lobula proper are here referred to as the lobula complex. Although the present study does not resolve important anatomical issues concerning the relative propinquities of the lobula-associated neuropiles, these structures do lie within the optic lobes and some are prominent in the overall pattern of diaphorase staining. They are therefore included in the present study, and we have elected to rename them by reference to the same topological axes (referenced to the embryonic neuraxis) used

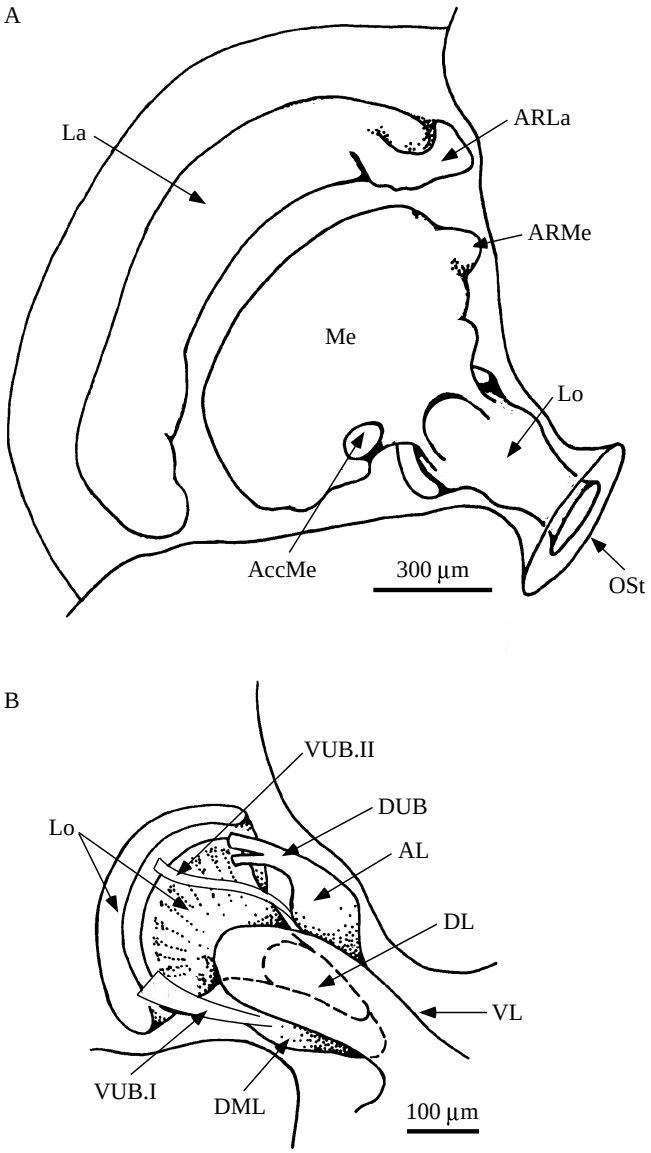

Fig. 2. Organisation of neuropiles in the optic lobe. (A) The three major neuropiles, the lamina (La), medulla (Me) and lobula (Lo), are shown. Subdivisions of the major neuropiles, the anterior rim of the lamina (ARLa), the anterior rim of the medulla (ARMe) and the accessory medulla (AccMe), are also shown. The position of the optic stalk (OSt), where the optic lobe joins the brain, is also indicated. (B) Neuropilar compartments of the lobula complex: lobula (Lo), dorsal lobe (DL), dorso-medial lobe (DML), ventral lobe (VL) and the anterior lobe (AL) with its associated dorsal uncrossed bundle (DUB). The two ventral uncrossed bundles VUB.I and VUB.II are also shown.

in defining the true optic neuropiles. Clearly, any future resolution of the question of their origin may then require a revision of the practical nomenclature adopted here.

In addition to the second optic chiasma, the medulla and lobula complex are also connected by uncrossed bundles of fibres (Gouranton, 1964; Rowell *et al.* 1977) that connect either the dorsal or the ventral face of the medulla to various parts of the lobula complex. The dorsal uncrossed bundle (DUB) connects the medulla and the anterior lobe (AL), and the two ventral uncrossed bundles (VUB.I and VUB.II) pass between the medulla and the dorso-medial lobe (DML).

Table 1. *Abbreviations*

AccMe	accessory medulla
A	anterior
AL	anterior lobe
ARLa	anterior rim of the lamina
ARMe	anterior rim of the medulla
Br	brain
CE	compound eye
D	dorsal
DL	dorsal lobe
DML	dorso-medial lobe
DUB	dorsal uncrossed bundle
EMe	external medulla
FG	frontal ganglion
IMe	internal medulla
La	lamina
LMC	lamina monopolar cell
Lo	lobula
LOc	lateral ocellus
Me	medulla
MOc	median ocellus
OCh.I	first optic chiasma
OCh.II	second optic chiasma
OL	optic lobe
OSt	optic stalk
RF	recurrent fibre
SF	single fibre
SL	serpentine layer
T	trachea
V	ventral
VL	ventral lobe
VUB.I	first ventral uncrossed bundle
VUB.II	second ventral uncrossed bundle

All anatomical terms and abbreviations used in this paper are given in Table 1. Figures show the right optic lobe from a ventral aspect unless otherwise stated.

Overview of NADPH-diaphorase staining in the optic lobe

An overview of some of the principal features of the diaphorase staining pattern in the optic lobe is provided by the photomontage of the whole of the right lobe sectioned horizontally (Fig. 3). A repeatable, extensive pattern of diaphorase staining that is locally intense and associated with identified tracts, distinct neuropiles and cell body groups is revealed. Perhaps the most striking feature of the staining is in the anterior lobe (AL) of the lobula complex and its connection by means of the dorsal uncrossed bundle (DUB) to the medulla. Within the lobula, some of the strata also stain strongly, notably in the central lobula region. Also immediately apparent are the stained neuronal components of the first optic chiasma (OCh.I), the lamina (La) and the projection of these elements to the external medulla (EMe). Less apparent, but nonetheless noticeable in Fig. 3, are several of the cell body groups (see

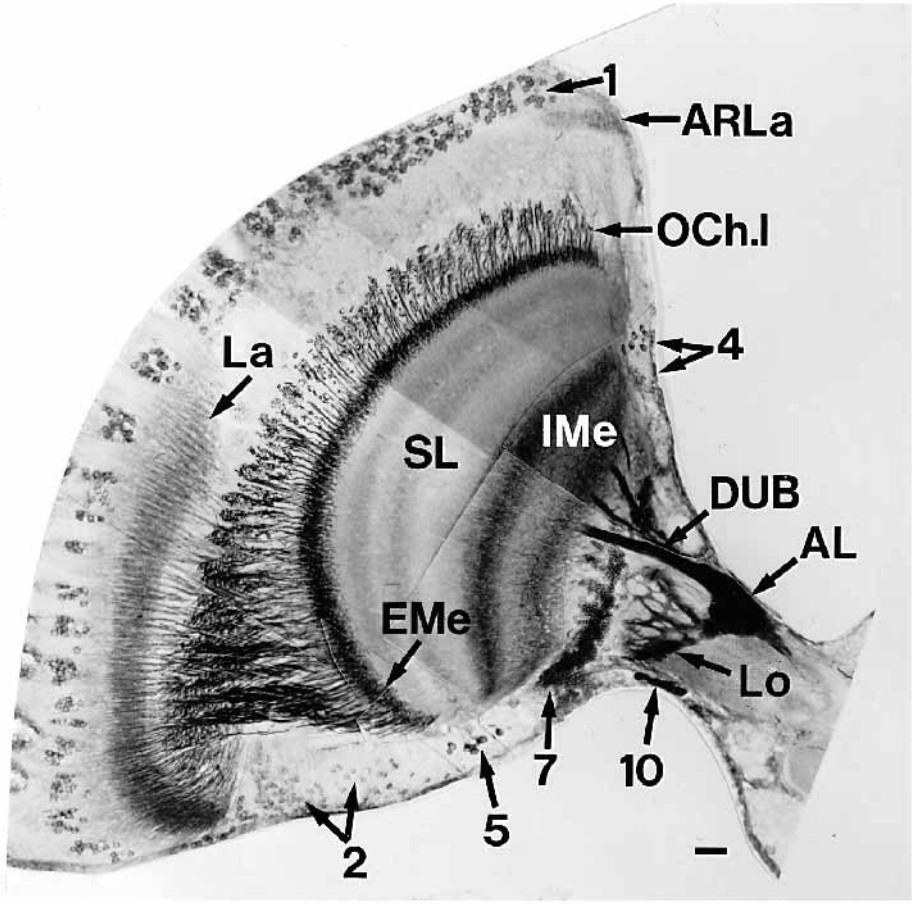

Fig. 3. Photomontage of NADPH-diaphorase staining in a longitudinal section of the right optic lobe. In the lamina (La), only large monopolar cells (group 1) and their axons are stained. These are responsible for the dense staining seen in the first optic chiasma (OCh.I) and for the band of staining in the external medulla (EMe). The plane of the section restricts a view of the axons of group 1 cells to the posterior half of the section. Two lightly stained layers in the serpentine region of the medulla (SL) and two strongly stained layers in the inner medulla (IMe) are also evident. Cells in the medulla that are stained are located along the anterior and posterior margins of the optic lobe (groups 4 and 5). Intense staining is associated with identified neuropiles of the lobula complex, especially in the lobula itself (Lo) and the anterior lobe (AL) with its associated dorsal uncrossed bundle (DUB). Identified stained cell body groups are indicated by the numbers (refer to Fig. 4). Scale bar, 70 μm.

below). Note that there appears to be no major diaphorase-positive tract connecting the optic lobe to other parts of the brain, notwithstanding the possibility that parts of the lobula complex may actually derive from midbrain structures.

Each of these features and the complete and consistent pattern of diaphorase expression in cell bodies and fibre tracts are described in further detail below.

Cell body groups

Providing an anatomical description of the population of diaphorase-positive cell bodies presents some difficulty because, with some notable exceptions (the lamina monopolar cells, for example), the cell bodies of the optic lobe have not been comprehensively classified. In general, therefore, it has not been possible to relate our findings to reliable existing maps of the optic lobe cell bodies. In order to provide a systematic description, we have therefore defined eleven groups of cell bodies that stain consistently and appear clearly to fall into natural clusters that are bilaterally symmetrical. Some of these groups appear to be homogeneous with respect to soma size and others are not. In either case, we do not intend to suggest that the neurones of a particular group are functionally equivalent. The classification provides a convenient descriptive framework that we hope will be a practical starting point for future investigations of the anatomy and histochemistry of the locust optic lobe. An indication of the location and boundaries of each group in the optic lobe is provided in Fig. 4A,B, and sample photomicrographs of cells belonging to each are shown in Fig. 5.

Although the intensity of staining of a group may be variable among individuals, it must be consistently above background level to be included in this description as a group. Also, the position and extent of a group with respect to the various optic neuropiles are defining qualities that are consistent. In Fig. 5, some cell bodies are visible (see Fig. 5C for example) and stained above background level but are not included as members of one of our groups. This is because staining in these cells is unreliable and not necessarily bilateral and, although significance may be attached to their staining, they cannot by our own restrictive definition at this stage be included in the anatomical analysis.

In a number of instances in the present study it has not been possible positively to associate cell bodies with their fibre tracts or arborizations, although in some cases evidence from Golgi preparations and other published anatomical accounts serve as useful guides. Also, it should be noted that, in defining cell body groups, we do not imply that neurones within a group necessarily share similar patterns of arborization.

The eleven bilaterally symmetrical groups of consistently stained monopolar cell bodies are as follows.

Group 1. This is a large (thousands) population of cell bodies (about 14 μm in diameter) of lamina monopolar cells

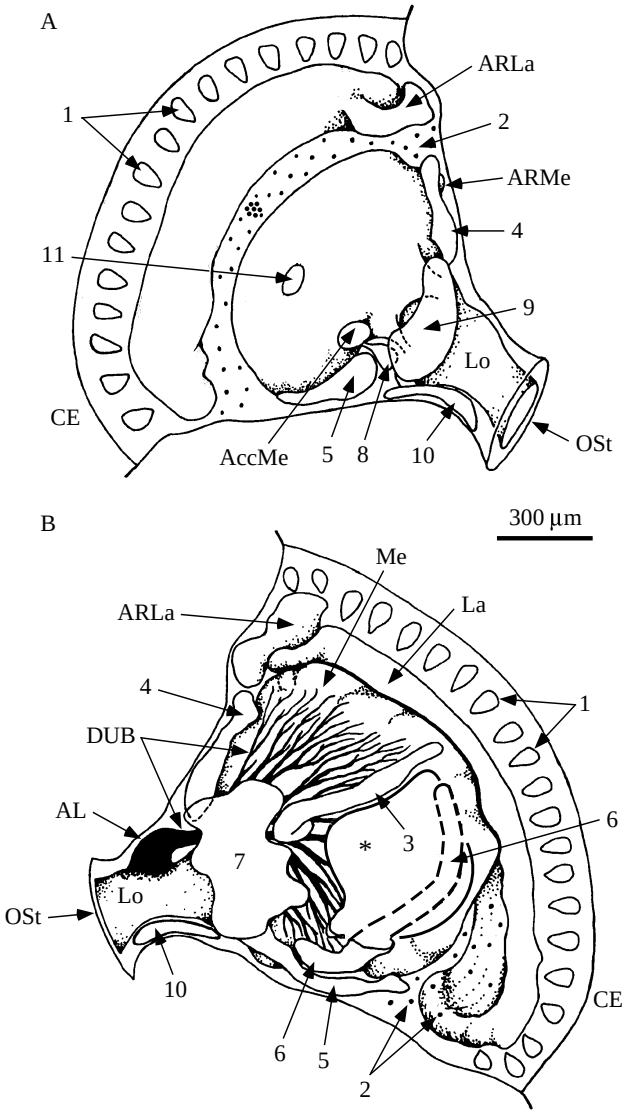

Fig. 4. Diagrams showing the distribution of the eleven numbered NADPH-diaphorase stained cell body groups in the right optic lobe. (A) Ventral aspect. (B) Dorsal aspect. The heavily stained anterior lobe (AL) and dorsal uncrossed bundle (DUB) are also shown as reference points. The asterisk indicates the position of cell bodies which are occasionally lightly stained (see also Fig. 5C). They are not assigned to a cell body group in this study because their staining is inconsistent.

arranged in rows running dorso-ventrally between the eye and the lamina parallel to the horizon in the visual field of the animal. The rows of group 1 cells are cut in Fig. 3 and therefore appear as clusters of cell bodies at the external margin of the lamina. Transverse sections through the rows of group 1 cell bodies are also evident in Fig. 4A,B. In a photomicrograph (Fig. 5A), the rows of group 1 cells can be seen separated by bundles of photoreceptor cell axons. Diaphorase-positive cells in this group are clustered amongst non-staining cell bodies of the lamina monopolar neurones and represent approximately 40 % of the total. This suggests that the group 1 diaphorase-positive lamina monopolar cell bodies account for more than one of the five species of monopolar cells in each cartridge. Analysis of monopolar neurone fibre projections through the lamina and of terminations in the medulla, however, show that there is only one diaphorase-positive morphological type (corresponding to the M1 type of large monopolar cell, see below and Fig. 6). This indicates that there are group 1 cell bodies that do not visibly contribute diaphorase-positive fibres to the lamina (see Discussion).

Group 2. This is a large (hundreds) population of lightly stained cell bodies (about 11 μm in diameter) distributed diffusely in the cellular cortex which surrounds the first optic chiasma. They are clearly visible in Fig. 3 and at a higher magnification of the same preparation in Fig. 5B. The position of these cell bodies suggests strongly that they are tangential lamina cells (Strausfeld, 1976). Weak staining of group 2 cell bodies has prevented positive identification of their arborizations. Within group 2, there is a weakly staining cluster of about 15–20 cells on the plane of the equator of the compound eye dorsally at the outer face of the medulla (Fig. 4A).

Group 3. This is a narrow band of about 220 cell bodies (about 13 μm in diameter) located on the plane of the equator dorsally above the second optic chiasma and immediately beneath the neural sheath (Figs 4B, 5C). Cells in this group probably contribute along with others in the cortex of the second optic chiasma to the dorsal uncrossed bundle or DUB (see below).

Group 4. Consisting of about 100 cell bodies (10–20 μm in diameter), this is a band of cells located along the anterior rim of the medulla (ARMe) which runs as far as the point of apposition of the medulla with the external capsule of the lobula. Cells in this group can be seen in Fig. 3 and the extent of the band and its relationship to other cell body groups can be seen in Fig. 4A,B. Axons arising from the group 4 cell bodies can be seen entering the medulla in Fig. 5D.

Group 5. This is a strip of about 120 cell bodies (8–20 μm in diameter) extending along almost the entire length of the distal posterior rim of the medulla and curving ventrally to the level of the accessory medulla (Figs 3, 4, 5B,E). Individual cells in this group are clearly visible in Fig. 3, and the boundary of the group is indicated in Fig. 4A,B. Axons arising from individual group 5 cells can be seen entering the medulla in Fig. 5B,E.

Group 6. This is a strip of about 100 cell bodies (8–20 μm in diameter) located along the proximal posterior rim of the medulla. The boundary of the group can be seen in Fig. 4B and individual cells in Fig. 5E.

Group 7. This is a layered dorsal plate of several thousand cell bodies (about 8 μm in diameter) which follows the curvature of the external capsule of the lobula. Cells of this group can be seen in Figs 3, 5B,C, and the group boundary is shown in Fig. 4B. The group 7 cluster is indented by cell body group 3 and periodically penetrated by the second optic chiasma, the DUB and various tracheae.

Group 8. This is a small, homogeneous group of about 25 densely stained cells (about 9 μm in diameter) located near the

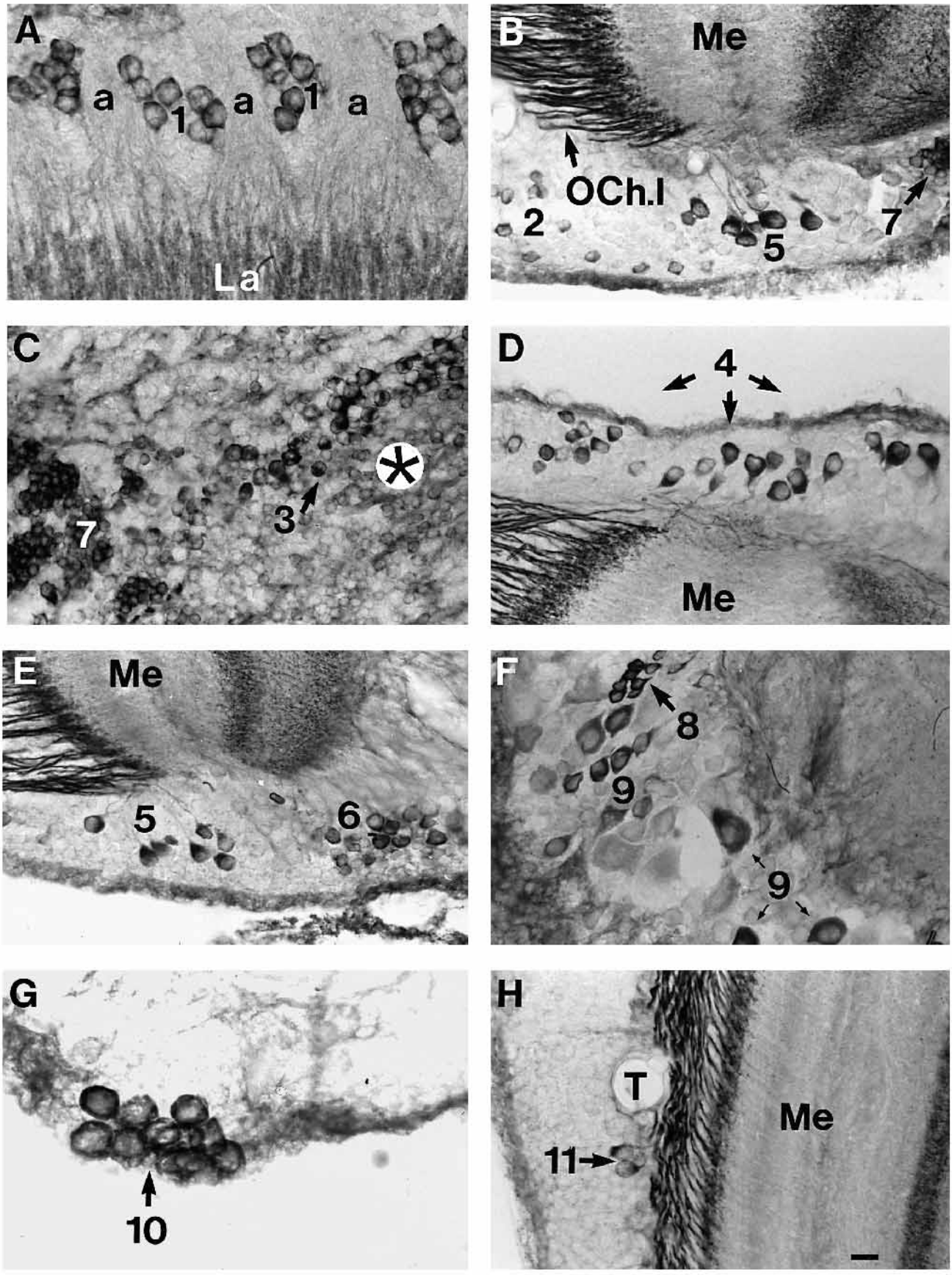

Fig. 5

accessory medulla (Fig. 4A, see also Fig. 10). Group 8 cells are clearly distinguished from the heterogeneous group 9 cells that are in close proximity (Fig. 5F). Group 8 cell bodies are intensely stained, tightly packed and homogeneously smaller than others nearby. Furthermore, they all project neurites as a small distinct bundle to the accessory medulla (see below).

Group 9. This is a scattered, heterogeneous population of cell bodies located directly ventral to the lobula (Figs 4A, 5F). Cells in this group vary considerably in size and some are quite large (12–35 μm in diameter).

Group 10. This is a tightly bunched plate of 17 large (22–34 μm in diameter) cell bodies that follows the posterior curvature of the junction between the optic lobe and the brain, within the optic stalk. The outline of the group is shown in Fig. 4A,B. These cells are clearly visible in Fig. 3, and the tight cluster is shown at higher magnification in Fig. 5G.

Group 11. This is a relatively inconspicuous group of four cell bodies (about 13 μm in diameter) located on the ventral surface of the medulla (Fig. 4A) near a major branch of the trachea (T) on the ventral surface of the optic lobe (see Burrows, 1980). Two members of the group 11 cluster are shown in section in Fig. 5H.

Fibre tracts and neuropiles

Here we will consider how constellations of stained neurones contribute to and connect between optic neuropiles. While the cell body groups described above must contribute to one or more fibre tracts and neuropile areas, often with considerable complexity, it has not always been possible to assign stained cell bodies to fibre tracts. This may be due to technical difficulties associated with reconstructing pathways or may suggest that the diaphorase reaction is not necessarily uniformly distributed within some neurones, being visible in the soma but not in the arborization for example.

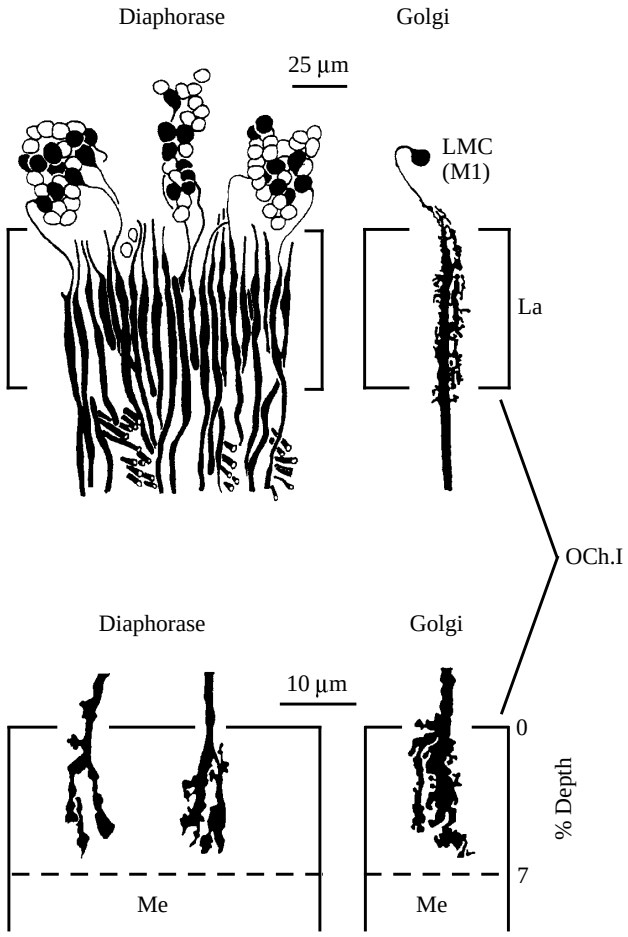

Fig. 6. Reconstructions of the projections of monopolar cells from the lamina (La) to the medulla (Me) as revealed by NADPH-diaphorase staining (group 1 cells in black) and Golgi staining. The NADPH-diaphorase terminations match those of Golgi-stained M1-type lamina monopolar cells (LMC) in being the largest, in ending in the first 7 % of medullary depth with bifid terminations and in having very limited branching in the lamina.

Fig. 5. Photomicrographs showing the eleven numbered NADPH-diaphorase-stained cell body groups in detail. All of the images are from horizontal sections unless stated otherwise. (A) Group 1 cell bodies of lamina monopolar cells. The positions of bundles of photoreceptor axons between clusters of the group 1 cells are indicated by the letter a. Note also the dense staining in the lamina (La), where synaptic input from the photoreceptor cells is received. (B) Group 2 cells located in the cellular cortex which surrounds the heavily stained first optic chiasma (OCh.I) comprising group 1 cell axons (arrow). Group 5 cell bodies can also be seen here with projections into the medulla (Me). Note also the group 7 cell bodies near the dorsal surface of the medulla (Me) (arrow). (C) Group 3 cell bodies which form a long narrow band just under the neural sheath on the dorsal side of the optic lobe (viewed from the dorsal aspect; see Fig. 4B). The asterisk indicates the position of cell bodies which surround group 3 cell bodies but have not been assigned to a cell body group because they stain inconsistently and weakly. Group 7 cells are also visible. (D) Group 4 cell bodies located in the anterior cellular cortex around the medulla (Me). (E) Group 5 and 6 cell bodies located in the posterior cellular cortex around the medulla (Me). (F) The small homogeneous cell bodies of group 8 can be seen clustered in close proximity to the heterogeneous and more dispersed cell bodies of group 9. (G) Transverse section showing group 10 cell bodies located on the posterior side of the optic stalk. (H) Parasagittal section showing two of the four stained cell bodies that form group 11 located ventrally near a major branch of a trachea (T). Scale bar, 15 μm (A) and 23 μm (B–H).

Lamina-to-medulla connection

The group 1 lamina monopolar cells (Fig. 3) provide the diaphorase-positive connection from the lamina to the medulla (Figs 6, 7). Approximately 40 % (42 % in a count of the 393 cells included in a section from the equator to the posterior margin of the lamina) of the monopolar cells of the lamina appear to be diaphorase-positive. They project from the outer face of the lamina into the lamina, one profile per lamina cartridge, where they attain a diameter of 5 μm, arborize and then pass *via* the first optic chiasma to the outer surface of the external medulla (Fig. 7B,C). The axons of these group 1 cells penetrate the outer face of the medulla to a depth of about

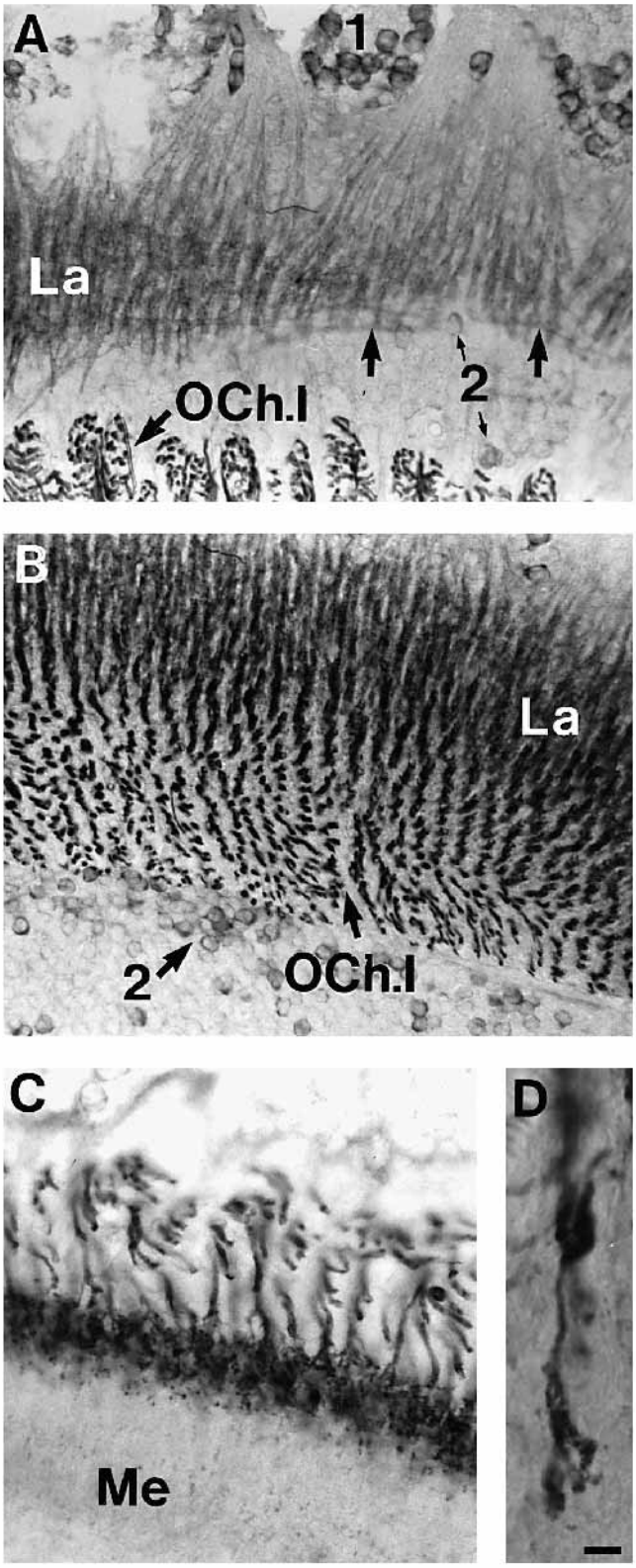

Fig. 7. Photomicrographs showing NADPH-diaphorase-stained elements in the lamina-to-medulla connection. (A) Faintly stained fibres (larger arrows) in the lamina resemble those of wide-field tangential cells (Strausfeld, 1976) and may be derived from group 2 cell bodies. Group 1 cells arborize in the lamina (La) and then project *via* the first optic chiasma (OCh.I) to the medulla. (B) Group 1 cell axons in the lamina and the proximal OCh.I. Note group 2 cell bodies. (C) Bifid terminations of group 1 cells form a distinct layer in the external layer of the medulla (Me). (D) High-power image of a stained bifid termination of a group 1 cell. Scale bar, 23 μm (A,B), 12 μm (C), 6 μm (D).

15 μm or approximately 7 % of the total depth of the medulla. Here, each terminates with a characteristic bifurcation (Fig. 7D). The terminations of all of the diaphorase-positive axons produce a distinct and dense diaphorase-positive layer at the outer face of the medulla (Figs 3, 7C).

There are five species of lamina monopolar neurones in the lamina, with one of each type being represented in each of the radial optic cartridges. Our estimate that approximately 40 % of the lamina monopolar cell bodies are diaphorase-positive clearly indicates that two cell types can be accounted for among the group 1 cells defined above. Analysis of the diaphorase-positive fibre projections in the lamina, however, suggests that only one cell type is represented here.

The position of the group 2 cell bodies suggests that they are tangential lamina cells (Strausfeld, 1976), which typically have a cell body in the cortex around the first optic chiasma and project to the medulla and the lamina, where they arborize. Owing to weak staining in group 2 cells, it has not been possible to trace these neurones but, nevertheless, there are stained fibres in the lamina that resemble those of wide-field tangential cells (Fig. 7A).

Medulla-to-DUB-to-lobula complex (anterior lobe) connection

The DUB and especially the anterior lobe (AL) of the lobula complex are the most outstanding features of NADPH-diaphorase staining in the optic lobe (Figs 3, 8A, 9A). The projection patterns of individual neuronal elements which contribute to the DUB have been described (Rowell *et al.* 1977). The DUB is composed of approximately 500 similar neurones with a small ellipsoid field of dendritic arborization on the proximal face of the internal medulla. Their cell bodies are located in the cellular cortex of the second optic chiasma and their axons project to the anterior lobe in groups, converging as tributaries to form the DUB.

Fibres running in the DUB appear to be of one type having an axon diameter of about 2 μm. Each fibre has extensive ramification in the anterior lobe, where they terminate (see also Rowell *et al.* 1977). The diaphorase-positive cell bodies that contribute to the DUB and the dense arborization in the anterior lobe must be among those that are found in the cellular cortex associated with the second optic chiasma. Reconstructions of Golgi-impregnated neurones that form the DUB indicate a homogeneous population of approximately 8 μm diameter cell bodies in a region consistent with the location of group 7.

Neuropile elements of the lobula complex

The lobula complex comprises several neuropilar subcompartments of which the anterior lobe, the dorsal lobe

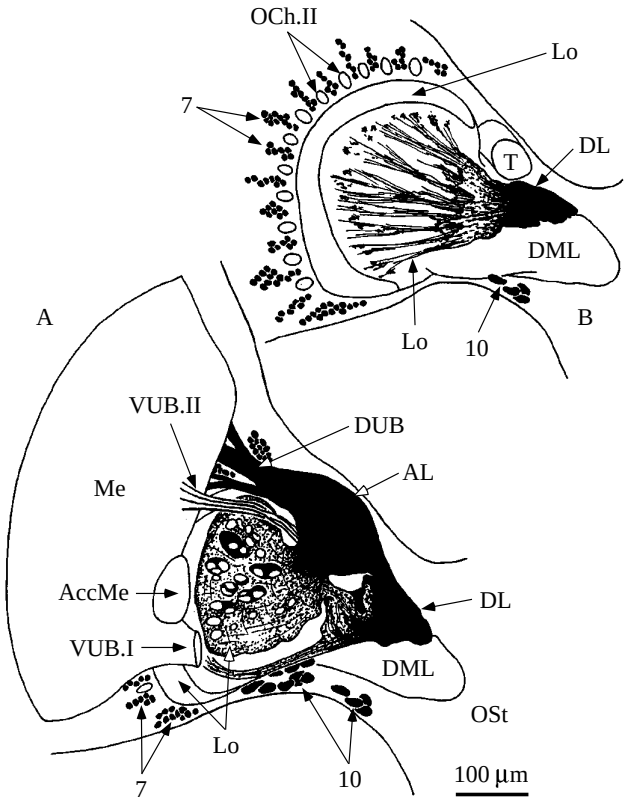

Fig. 8. Reconstruction of NADPH-diaphorase-stained structures in the lobula complex of the optic lobe showing the relative intensity of staining in lobula complex neuropiles. (A,B) Two contiguous reconstructions of the lobula complex in which A is ventral to B. Note the intense staining in the dorsal lobe (DL) and the anterior lobe (AL) with its associated dorsal uncrossed bundle (DUB). NADPH-diaphorase staining in the lobula (Lo) reveals ray-like fibre bundles dorsally (B) and a lacunate structure ventrally (A). The lacunae contain the projections of transmedullary cells. Stained cell body groups 7 and 10 and other anatomical structures (Me, OCh.II, AccMe, DML, VUB.I, VUB.II, OSt) are shown as reference points. T, trachea.

and the proximal region of the lobula proper show strong diaphorase staining (Figs 8, 9). The dorso-medial lobe (DML) and the outer strata or distal region of the lobula also stain, but far less densely. The anterior lobe (AL), with its associated dorsal uncrossed bundle (DUB), collects fibres (of group 7 cells) from the tributaries of the DUB in the medulla and constitutes the most prominent feature of the lobula complex (see above).

The lobula shows a complex pattern of staining revealing a lacunate structure ventrally (Figs 8A, 9B) and a fan-like arrangement of fibre bundles dorsally (Fig. 8B). Within the lacunae are the unstained projections of bundles of transmedullary cells. Staining in the dorsal lobe is denser and more uniform than in the lobula proper. This staining is derived from fibres of the tangential fan arrays that are a feature of one stratum of the dorsal part of the lobula.

It is difficult to be sure about the origin of the neuropile staining in the lobula and the dorsal lobe of the lobula complex, but Golgi preparations show that the lobula consisted of

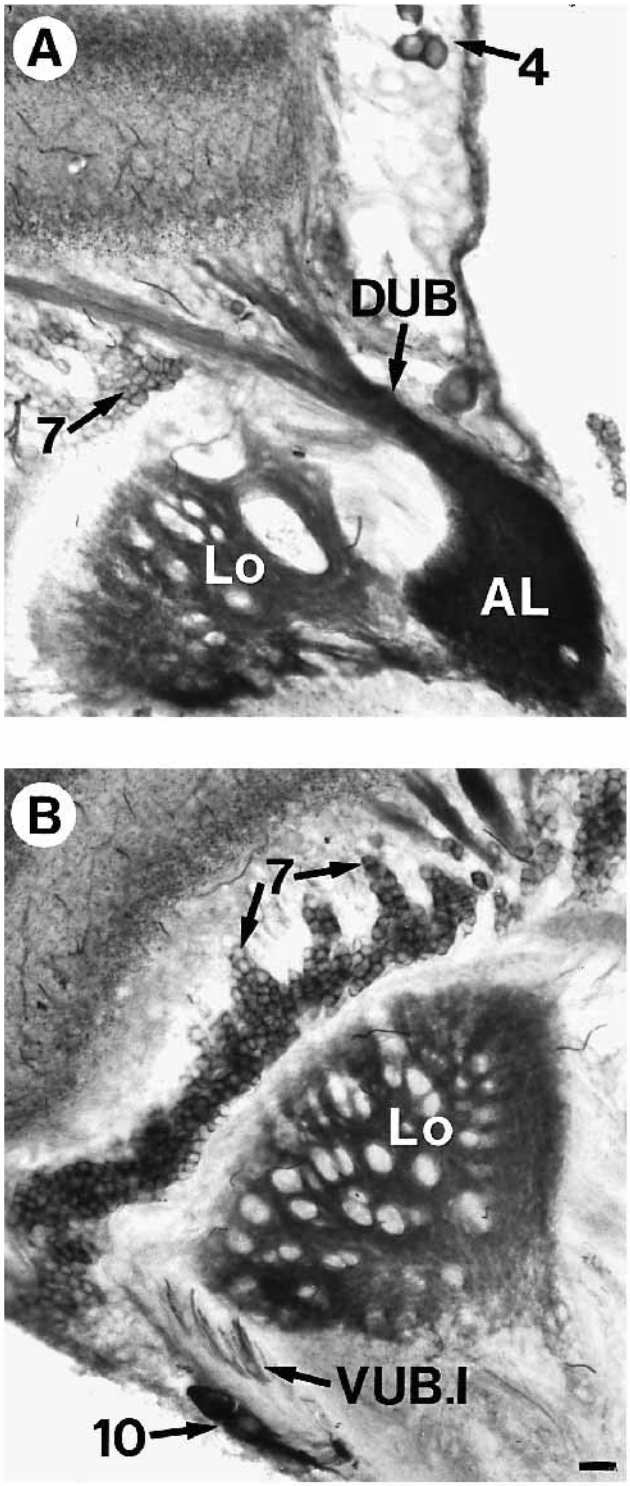

Fig. 9. Photomicrographs showing NADPH-diaphorase-stained structures in the lobula complex. (A) Staining in the anterior lobe (AL), dorsal uncrossed bundle (DUB) and the lobula (Lo). Note also the stained group 4 and group 7 cell bodies. (B) Staining in the lobula (Lo) and the first ventral uncrossed bundle (VUB.I). Note also the group 7 and group 10 cell bodies. Compare with Fig. 8. Scale bar, 25 µm.

columnar neurones with cell bodies in the second optic chiasma following the curvature of the outer surface of the

lobula. These neurones do not project to the dorsal lobe, indicating that the lobula staining is derived from group 7 cells and that the dorsal lobe receives a projection from a different, as yet unidentified, population of neurones.

The distal or outer strata of the lobula contain some diaphorase-positive fibres and these probably derive from the same columnar neurones that penetrate the distal part and project to varying levels deeper in the lobula. Light staining is also found in the dorso-medial lobe and this is derived from the terminal arborizations of group 10 cells (see below) and from centrifugal projections of a small number of neurones from the brain protocerebrum (Figs 10, 11B).

Lobula complex-to-medulla interconnection

The major connection between the medulla and the lobula complex is provided by the DUB and is described above. All other diaphorase-positive connections appear to be provided by the 17 neurones of cell body group 10. Fibres arising from group 10 cell bodies project to a point of bifurcation from which two conspicuous fibre bundles emerge (Fig. 10). One of these contributes to one of the ventral uncrossed bundles (VUB.I) which projects to the medulla (Figs 10, 11A,C). The other gives rise to arborizations in the dorso-medial lobe (Fig. 11B). From there, a number of fibres project in the direction of the medulla by three different routes. First, as an uncrossed bundle of just four fibres which we have defined as VUB.II (Fig. 11C). This bundle runs in the cleft between the anterior lobe of the lobula complex and the lobula proper to penetrate the medulla at the same level as the VUB.I, but anteriorly. The second route is followed by only a single fibre (Figs 10, 11C), which runs dorsal to the anterior lobe to enter the anterior ventral medulla. The third route is followed by recurrent fibres (RF) arising from the dorso-medial lobe and running singly in one of the bundles of the second optic chiasma. It is not clear whether the third group of fibres reaches the medulla, but these fibres do give rise to fine arborizations in the outer stratum of the distal lobula. It is clear, however, that most fibres from group 10, *via* the VUB.I, contribute to fine arborizations in two conspicuous bands of the internal medulla, and some fibres from group 10 contribute to fine arborizations in much of the outer stratum of the lobula.

Other contributions to the medulla

In addition to the contributions to the medulla described above, fibres from several other stained cell body groups penetrate the main body of the medulla at various levels, but most do not reveal a clear picture with respect to their projection patterns. For example, fibres from cell body group 4 project into the main body of the medulla and centrifugally to the anterior rim of the lamina. Most conspicuously, however, there is a contribution of initial arborizations from fibres of cell body group 8 in the accessory medulla (Figs 5F, 10). Fibres from this group then project tangentially into the medulla at various depths.

Optic-lobe-to-brain connection

Notwithstanding the likely protocerebral origin of parts of the non-retinotopic lobula complex, no conspicuous fibre projections from the optic lobe to the main brain were observed. Some weakly stained fibres are, however, present in the DML and probably originate in the protocerebrum (Figs 10, 11B).

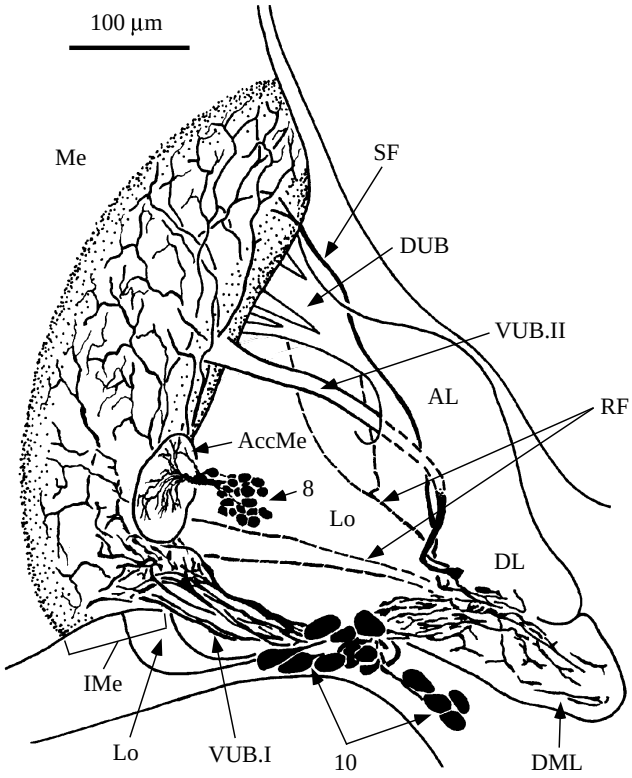

Fig. 10. Reconstruction of NADPH-diaphorase-stained elements that contribute to the lobula complex-to-medulla connection. Fibres arising from the group 10 cell bodies project to a bifurcation point from which two fibre bundles emerge: one contributes to VUB.I and the other gives rise to arborizations in the DML. From the DML, a number of fibres project towards the medulla *via* three routes: first, as a bundle of four fibres (VUB.II); second, as a single fibre (SF); and third, as recurrent fibres (RF) running singly in one of the bundles of OCh.II (see also Fig. 11). The fibres from group 10 cells contribute to fine aborizations in the internal medulla (IMe).

Discussion

We have shown that the optic lobe of the locust consistently contains an extensive population of NADPH-diaphorase-positive neurones that make important contributions to the major optic neuropiles, the lamina, medulla, lobula and lobula-associated neuropiles. No other single histochemical method has revealed the full pattern of staining that we describe here. Diaphorase therefore appears to be associated with a neurochemically distinct and previously undescribed architecture in the insect optic system. The functional significance of this is unknown, but the results are indicative of an important role in insect vision for NO since, in the locust CNS as well as in other invertebrates and in mammals, diaphorase can be a specific histological method for the

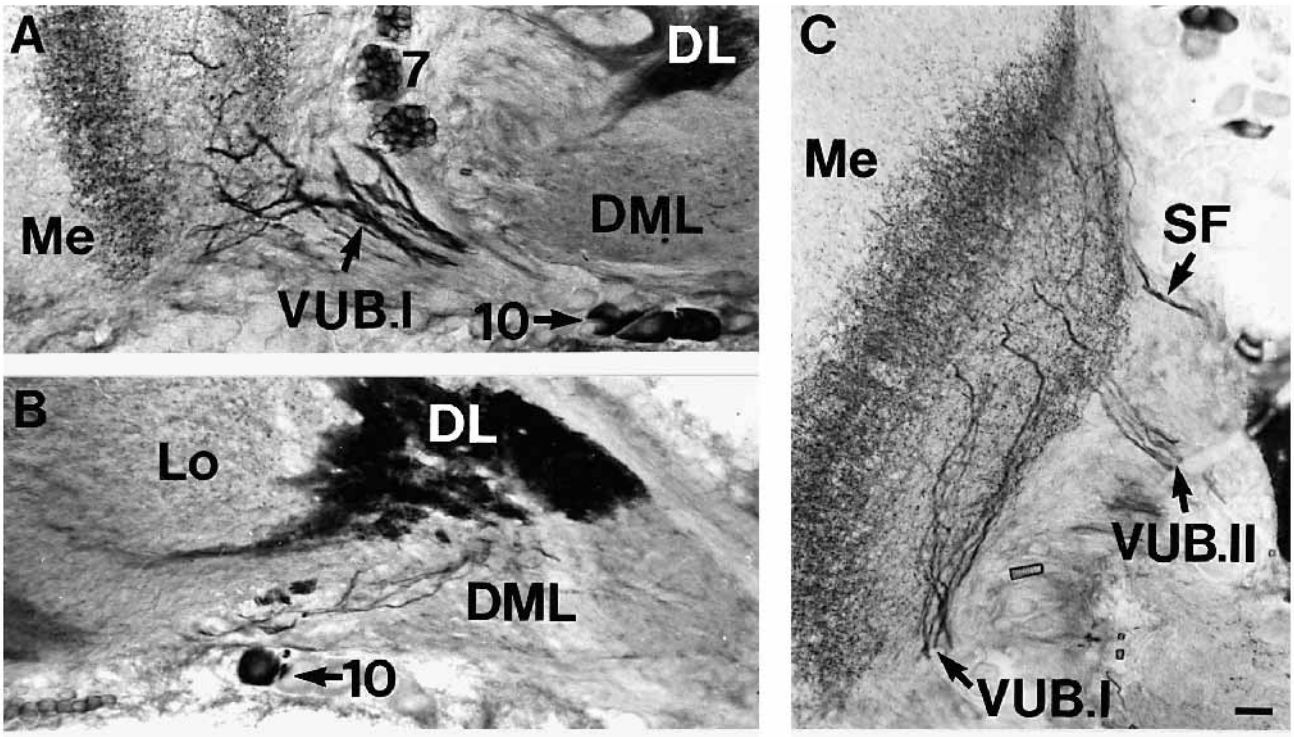

Fig. 11. Photomicrographs showing NADPH-diaphorase-stained elements that contribute to the lobula complex-to-medulla connection. (A) Stained fibres derived from group 10 cell bodies contribute to VUB.I and arborize in the internal layer of the medulla (Me). (B) Stained fibres derived from group 10 cell bodies project to and arborize in the dorso-medial lobe (DML). Note also the intense staining in the dorsal lobe (DL) compared with the more diffuse staining in the lobula (Lo). (C) Stained group 10 projections to the internal layer of the medulla *via* VUB.I, the four fibres of VUB.II and as a single fibre (SF). Scale bar, 23 μm.

localization of NOS (Dawson *et al.* 1991; Hope *et al.* 1991; Matsumoto *et al.* 1993). Specifically, in the locust CNS, diaphorase staining for NOS has been verified by measuring enzymatic activity in different parts of the brain using both the citrulline synthesis assay and an assay of NO production (Elphick *et al.* 1995). The highest levels of NOS activity ($0.7\,nmol\,NO\,mg^{-1}\,protein\,min^{-1}$) were found in regions showing the highest intensity of diaphorase staining.

If it is accepted that the diaphorase staining in the optic lobes is due to the presence of neuronal NOS, as we have argued, then it is reasonable to speculate on the possible function of NO in the processing of visual information. In general terms, NO has physical properties not shared by other transmitter molecules in the optic lobe and these may allow us to draw some general conclusions about the context in which it might function in vision. For example, as NO is a gas, it will diffuse freely in three dimensions from the site of synthesis. It does not seem to be well suited therefore to a role in topological representation of the visual world, and so we consider it unlikely that NO signalling is involved in high-acuity pattern recognition or image analysis. Likewise, the requirement in the NO–cyclic GMP signalling pathway for enzyme activation, diffusion, second messenger production and second messenger action would seem to rule out functions in vision that require very rapid processing. These considerations lead us to suggest that we might expect NO signalling to be involved in such processes as gain control, especially where the sensitivity of the visual system over broad areas of the visual field is altered over longer periods.

But is it possible to be more specific about the likely functional role of diaphorase-positive neurones in the optic pathway? In this we must be cautious and it is important to recognise the distinction between ascribing a function to a pathway or projection and defining a role for NO in that function. This is because it is likely that the diaphorase-positive neurones employ more than one chemical signalling system. There is, therefore, not necessarily a simple correspondence between the physiological function of the neurones and the role of NO in that function.

With these caveats in mind, we are nevertheless struck by the consistent and dense diaphorase staining displayed by the DUB and its continuation into the anterior lobe of the lobula complex. This is interesting because neurones of the DUB have a known specific function in locust vision. The DUB and anterior lobe form part of a network of neurones involved with the responsiveness of a prominent and identified visual interneurone, the lobula giant movement detector or LGMD (O'Shea and Williams, 1974; O'Shea and Rowell, 1975; Rowell *et al.* 1977). While the LGMD is not diaphorase-positive, the presence of strong diaphorase staining in the neurones that form the DUB strongly suggests a role for NO in shaping the response characteristics of the LGMD. The

LGMD was the first identified interneurone in the optic lobe in the locust (O'Shea and Williams, 1974) and is responsive to movements of small targets in the entire receptive field of the ipsilateral eye. It is insensitive to large-field motion and is inhibited by whole-field dimming and brightening. In addition, an important characteristic of the responsiveness of the LGMD is plasticity. For example, it shows rapid habituation or response decrement to repeated movement or brightening and dimming of small targets (O'Shea and Rowell, 1976). Furthermore, it can be sensitised or dishabituated by strong non-visual arousing stimuli (Rowell, 1971).

Intracellular recordings from the LGMD before and after lesioning the DUB revealed a role for this pathway in mediating the inhibition of the LGMD response to whole-field stimuli (Rowell *et al.* 1977). One of the dendritic arborizations of the LGMD, the C field (see O'Shea and Williams, 1974), receives inhibitory, feedforward input from the constituent neurones of the DUB. It is possible that NO signalling by DUB neurones has a role in this feedforward inhibition of the LGMD and, moreover, this question ought to be amenable to direct experimentation. If NO is shown to be involved in feedforward inhibition, it suggests that its role may not be confined only to long-term modulatory effects. However, the inhibitory postsynaptic potential (IPSP) generated by an instantaneous whole-field dimming can have a duration of 200–300 ms and is not, by the standards of the visual system, a rapid processing event.

Plasticity, in particular habituation and dishabituation, in the LGMD is due to events at the lobula–medulla interface, where the main dendritic arborization of the LGMD, the A field, receives its excitatory input (O'Shea and Rowell, 1976). Diaphorase-positive fibres are present here, though generally we have not been able to reveal a clear picture that relates particular projection patterns to particular cell body groups. An exception to this is provided by the group 10 cell bodies. These give rise to a recurrent pathway that projects from proximal regions of the lobula complex to the lobula–medulla interface, producing fine arborizations in the distal lobula and the internal medulla. A centrifugal pathway of this kind would be suited to providing the whole-field, long-lasting sensitising or dishabituating feature of LGMD responsiveness. Indeed, NO signalling would seem to be suited to such a role (see above) and the anatomical evidence indicates that the biochemical pathway serving this role is present in the appropriate position in the visual system. Recently, octopamine has been implicated in the process of LGMD dishabituation (Bacon *et al.* 1995), but generally very little is known about the pharmacology of plasticity in the insect visual system, and other mechanisms are not ruled out. Considering the proposed role of NO in forms of synaptic plasticity in the mammalian brain (Schuman and Madison, 1994), it is intriguing to find evidence for NOS-containing neurones well placed to have a role in lability in the insect visual system.

The accessory medulla receives a very conspicuous contribution from the diaphorase neurones of group 8 (Fig. 10). This small neuropile area has been proposed as the site of the circadian pacemaker in the cockroach (Stengl and Homberg, 1994), and this raises the possibility that the discrete cluster of diaphorase-positive neurones innervating the accessory medulla has a role in circadian rhythm generation. Investigation of the part played by NO signalling in this function, however, is likely to be quite difficult since the most direct approach would involve localised and real-time measurement of NO generation *in situ* in the accessory medulla. Nevertheless, the hypothesis that NO is involved in circadian pacemaker regulation might also be testable by measuring circadian fluctuations in nitric oxide synthase activity using the well-established enzyme assay (Elphick *et al.* 1995).

The group 1 cells are of particular interest because they represent the most obvious correlate between diaphorase-positive profiles and a well-characterised cell type, namely the lamina monopolar neurones. Individual fibres traced from them cross the lamina in the optic cartridges and terminate in the outer face of the medulla, where they form characteristic bifid arborizations. All fibres we have been able to trace terminate in the same way and form a distinct diaphorase-positive band in the outer medulla stratum. Each optical cartridge in the lamina includes five axons, but only one is diaphorase-positive, always having the largest diameter and showing very limited tangential branching. These neurones almost certainly correspond to the largest class of lamina monopolar cell or M1, as described by Nowel and Shelton (1981). Thus, it appears that diaphorase staining marks an anatomically distinct fibre in the lamina representing a single species of lamina monopolar neurone. We observe, however, that about 40 % of the total number of lamina monopolar cell bodies appear to be diaphorase-positive. As each optical cartridge in the lamina includes one axon from each of five (M1–M5) types of monopolar cell (Nowel and Shelton, 1981), this observation indicates that about half of the fibres arising from the group 1 cell body population cannot be traced to the lamina. Also, as the 'missing' fibres represent about one-fifth of the total, it is tempting to speculate that one additional member of the five classes is also diaphorase-positive, though not detected. In the locust, Nowel and Shelton (1981) show that, among the five classes of lamina monopolar fibre, only M1 and M2 exceed 3 μm diameter and that the M1 fibre is significantly larger than M2, reaching 6 μm in diameter. The fibres of M3, M4 and M5 are distinctly narrower (<1.5 μm) and thus might be very difficult to trace and detect if any were weakly diaphorase-positive. Our results suggest, therefore, that diaphorase staining distinguishes the M1 positive from the M2 negative large monopolar cells and that there may be an as yet undetected diaphorase fibre type, presumably among the smaller M3–M5 classes. It is interesting that M1 and M2 are distinguished by diaphorase staining because in other respects they are very similar and the M2 fibres also have bifid terminations in the medulla (James and Osorio, 1996), though at somewhat deeper levels. Future physiological studies may reveal a role for NO in determining differences between the functions of the two largest monopolar neurones in locust vision.

This study has revealed an elaborate architecture of putative NOS-containing neurones in the optic lobe of the locust. We believe this suggests an important role for NO in insect vision. The diaphorase anatomy, in conjunction with the known function of some of the visual units and pathways, points to experimentally tractable systems in which the specific role of NO in vision can be assessed.

We are grateful to colleagues at Sussex, especially Danny Osorio, and Professor Karl-Ernst Kaissling at Seeweisen for helpful criticism and encouragement. This work was supported by Grant GR/J 33234 from the BBSRC and by the Max Planck Society.

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