Ultrastructure of the Synaptic Terminals of the Dorsal Giant Serotonin-IR Neuron and Deutocerebral Commissure Interneurons in the Accessory and Olfactory Lobes of the Crayfish

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ABSTRACT

The olfactory and accessory lobes in the crayfish are large spherical neuropils found on each side of its brain. The olfactory lobes receive the afferent axons of chemoreceptors that are located along the outer branches of the biramous first antennae. The accessory lobes receive a large input from interneurons whose axons lie in the deutocerebral commissure. A pair of large serotonergic neurons (the dorsal giant neurons) branch unilaterally in the accessory and olfactory lobes of each side. From physiological recordings, it has been proposed that the deutocerebral commissure interneurons synapse with elements in the accessory lobes that in turn connect to the dorsal giant neuron. It has also been proposed that the dorsal giant neuron is activated by inputs in the accessory lobe and that its output is in the olfactory lobe. This ultrastructural study tests this hypotheses by examining the polarity of synaptic terminals on dorsal giant neurons and deutocerebral interneurons that have been filled with neurobiotin. In double-labelled preparations, we found the deutocerebral interneurons to be presynaptic to elements in the accessory lobes, but none of these postsynaptic elements was identifiable as the dorsal giant neuron. The dorsal giant neurons receive many more synaptic inputs in the accessory lobes than in the olfactory lobe. Very few giant serotonin neuron output synapses were found in either lobe.

Indexing terms: crustacea, central nervous system, olfaction, serotonin, synapses

Freshwater crayfish possess an olfactory system consisting of rows of specialized chemoreceptive sensilla on the antennules that project exclusively to discrete areas of glomerular neuropil in the olfactory lobes of the brain. Adjacent to the olfactory lobes, and evidently also part of the olfactory system, are an additional pair of large glomerular neuropils, the accessory lobes. Four groups of interneurons are known to be associated with the olfactory and accessory lobes: (1) local interneurons with small somata (in cluster 9, see Sandeman et al., 1992) and with terminals in both the olfactory and accessory lobes (Mellon and Alones, 1993); (2) projection neurons with small somata (in cluster 10) whose axons extend in a single large tract (olfactory globular tract) from the olfactory and accessory lobes to the hemiellipsoid body in the lateral protocerebrum; (3) midbrain cells with larger somata (in cluster 11), whose axons terminate predominantly in the olfactory lobe; and (4) deutocerebral commissure neurons with small somata (also in cluster 11), with axons in the deutocerebral commissure and their terminals in the accessory lobe glomeruli (Sandeman and Luff, 1973; Arbas et al., 1988; Sandeman et al., 1992, 1993; Mellon and Alones, 1993).

Like other arthropods, crayfish have serotonin-immunoreactive (serotonin-IR) neurons that branch extensively among the olfactory lobe glomeruli, where they may exert a modulatory effect on the olfactory processing (Nässel and Elekes, 1984; Sandeman and Sandeman, 1987; Schmidt and Ache, 1992; Sun et al., 1993). Among the serotonin-IR neurons projecting to the crayfish olfactory lobes are a pair of very large neurons, one on each side of the brain, the dorsal giant neurons (DGNs; Sandeman and Sandeman, 1993).
Each DGN in the crayfish appears to end in all of the glomeruli of both the accessory and olfactory lobes, and each also sends a small branch that ramifies among the axons of the olfactory globular tract (OGT), which carries the output of the accessory and olfactory lobes (Mellon et al., 1992).

Physiological and anatomical evidence suggests that the input to the DGN is through its terminals in the glomeruli of the accessory lobe, and it has therefore been concluded that its output is made in the olfactory lobe (Sandeman and Sandeman, 1994). The glomeruli of the accessory lobe also contain the endings of projection neurons and local interneurons and of the interneurons whose axons run in deutocerebral commissure (DC). The DC interneurons receive unilateral inputs from a number of areas in the protocerebrum and terminate bilaterally in the accessory lobes. It has been proposed that they constitute an important input to the accessory lobes and thus to the olfactory system (Sandeman et al., 1995).

Anatomical confirmation of the direction of information flow within this circuitry is critical to any interpretation of the function of the accessory lobes and their relationship to the olfactory system. The aim of this study was to examine the ultrastructure of the terminals of DGN and DC interneurons in the accessory and olfactory lobe glomeruli to determine the polarity of the synapses of these neurons in these neuropils. To achieve this, the neurons were injected with neurobiotin to make their neurites visible in the electron microscope. We also examined DC interneuron terminals in the protocerebrum and deutocerebral commissure neuropil (DCN) and the DGN terminals in the olfactory globular tract neuropil (OGTN).

We find many more input than output synapses on the unilateral projections of the DC interneurons within the protocerebrum and DC neuropil, and numerous outputs as well as some inputs on their bilateral projections to the accessory lobes, confirming the hypothesis that these neurons constitute a major input to the accessory lobes (Sandeman et al., 1995). The DGNs receive many input synapses, not only within the accessory lobes and the OGTN but also in the olfactory lobes. They make few conventional output synapses from any part of their arborisation, which suggests that much of the release of serotonin is nonsynaptic. When DGN and DC interneurons were stained in the same preparations, no direct connections were found between them in the accessory lobe glomeruli, which share. Although we cannot exclude the possibility of direct DC interneuron to DGN contacts, their absence in electronmicrographs does explain the physiological responses that indicate the presence of at least one neural element between the DC interneurons and the DGNs (Sandeman et al., 1995). The presence of reciprocal connections in the accessory lobes between DC interneurons and so-far unidentified interneurons that had been predicted by physiological experiments was also confirmed by electron microscopy.

**MATERIALS AND METHODS**

Freshwater crayfish, *Cherax destructor*, were obtained from open air fish-breeding ponds near Sydney and kept in aquaria in the laboratory. They were fed on carrots, apples, and ground meat. Animals of both sexes with a carapace length of 4.5–4.8 cm were used in this study.

**Filling neurons with neurobiotin**

To gain access to the brain, we used the isolated head preparation described in detail elsewhere (Mellon et al., 1992; Sandeman et al., 1995). Illumination of the surface of the desheathed brain allows the large cell bodies of the DGN neurons to be visualized and penetrated with a microelectrode. The DGN is a large cell with extensive branches and is difficult to fill completely unless the electrode tip is broken back to a resistance of between 1 and 5 MΩ. Electrodes were filled with 5% neurobiotin (Molecular Probes) in 1 M KCl. Once in the cell, such electrodes seal into the membrane and will remain in place for several hours. The DGN was filled using 500-ms pulses of 10–20 nA delivered at 1 Hz for 30 minutes.

The cell bodies of the DC neurons also lie on the dorsal surface of the brain but are small, and their very narrow primary neurites present a significant barrier to the entry of the neurobiotin. This also effectively prevents action potentials from reaching the cell body and thus the physiological characterisation of neurons impaled in the soma. For this reason, the axons of the cells were penetrated where they cross the midline, directly beneath the point where the dorsal blood vessel enters the brain. The cells could therefore be characterised physiologically before dye injection. Those chosen for this study belonged to a class that respond to photic stimulation of the eyes (Sandeman et al., 1995). The application of 1–2 nA, 500-ms pulses of current delivered at 1 Hz for 30 minutes was usually enough to fill the cells. In preparations in which DC and DGN neurons were stained, this was done sequentially.

**Fixation and preparation for electron microscopy**

After filling the neurons, the head capsule was fixed for 1 hour at room temperature on a shaker by immersing it in freshly prepared fixative composed of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.15 M sucrose. Brains were then dissected out of the head capsule in the same sucrose-containing buffer, cut in half, attached directly to a cork base with cyanoacrylic glue, and sectioned at 50 μm on a Vibratome. Sections were fixed for another 30 minutes at room temperature on the shaker. They were then washed for 10 minutes in phosphate buffered sucrose and placed for 30 minutes in 50% alcohol to enhance penetration of the agents used for visualising the neurobiotin (Llewellyn-Smith et al., 1989). The sections were then washed again in the same buffer and incubated for 3 hours at room temperature on the shaker in a solution of horseradish peroxidase–conjugated avidin neutralite (Molecular Probes) diluted 1:100 in phosphate buffer. Sections were then intensified and developed using 3,5-diaminobenzidine following the method of Watson and Burrows (1981).

The sections were postfixed in 1% osmium tetroxide in distilled water for 30 minutes and en block stained in 2% aqueous uranyl acetate. The slices were then dehydrated and flat embedded in araldite between sheets of cleared electron microscopic film. The sections were examined and photographed. Selected areas containing labelled glomeruli or neuropil areas were cut out and remounted on araldite stubs, sectioned, stained with uranyl acetate and lead citrate, and examined in a Hitachi 7000 electron microscope.

**RESULTS**

**Dorsal giant neuron: Overview**

The morphology of the brain of *Cherax destructor* has been described in detail (Sandeman et al., 1992); we provide only a description of those areas of the brain that relate
directly to this study, all of which can be seen in the horizontal sections in Figure 1 and the diagram in Figure 2.

The paired olfactory lobes (OL) lie on each side of the brain. Their glomeruli are columnar (Fig. 1c,d), divided into an inner and outer portion, and radiate out from the centre of the lobe. The afferent fibres from the chemoreceptive sensilla on the antennule enter the brain from the ventral side and spread over the periphery of the olfactory lobe.
Large numbers of fine axons project from the centre of the lobes to fuse into a single tract, the olfactory globular tract (OGT), that passes medially and then anteriorly through the brain to end in the hemiellipsoid body in the lateral protocerebrum. The olfactory globular tracts from the two sides form a prominent medial chiasm in the centre of the brain. Two clusters of small cell bodies, one medial and one lateral to the olfactory lobe, contain the somata of local (LN) and projection (PN) neurons associated with the olfactory and accessory lobes.

Posterior to the olfactory lobes lie the larger paired accessory lobes (AL), which contain many small spherical glomeruli. Fine fibres that project from the centre of the accessory lobes join those from the olfactory lobes within the OGT. Like the olfactory lobes, the outer surfaces of the accessory lobes are also surrounded by bundles of fibres. These are branches of interneurons that have their axons in the deutocerebral commissure (DC). All axons in the DC have branches in one or other of the paired ventrally situated neuropils, the deutocerebral commissure neuropils (DCN).

The projections of the dorsal giant interneuron (DGN) occupy the entire accessory lobe and the inner portion of the olfactory lobe glomeruli (Fig. 3). Light micrographs of neurobiotin-filled DGNs show a number of parallel fibres that project from the primary neurite into the accessory lobe and that branch and terminate in fine (less than 1 μm in diameter) glomerular branches. Within the olfactory lobe, the terminals of the DGN are also of small diameter and are clustered within the columnar glomeruli. Branches of the DGN in the olfactory glomerular tract are diffuse and penetrate deep among the axons of the tract, where they branch among small, isolated, lens-shaped islands of neuropil, which constitute the olfactory glomerular tract neuropil (OGTN; Fig. 7).

Neurobiotin labelling of individual axons in the DC has revealed that the DC neurons fall into five distinct classes according to the projections of their branches and their response to illumination of the eyes and electrical stimulation of the second antennae (Sandeman et al., 1995). All classes of DC interneurons project bilaterally into the accessory lobes and unilaterally into the DC neuropil (DCN; Fig. 2). The DC contains two separate bundles of axons with diameters of 10–16 μm and 3–5 μm, respectively. Although both groups of axons project to the DC neuropils, the fine axons end in the lateral one-half of this neuropil and the coarse axons in the medial one-half (Fig. 4).

**Synaptic terminals in the central nervous system of crustaceans**

Our interpretation of a connection between the neurons in the olfactory and accessory lobes and the direction of information flow is determined by the identification of synaptic junctions between them. The criteria we have used to recognise synaptic junctions are those currently used for arthropods, namely, the presence of closely apposed, electron-dense membranes, a presynaptic electron dense body that is round or barlike and usually surrounded by clear or sometimes granular vesicles. Although the physiological confirmation that these structures are synapses is difficult in central nervous tissue, the physiologically characterised synaptic contacts between crayfish motorneurons and muscle (Govind et al., 1995) are ultrastructurally virtually identical to those found between central neurons.

**Terminals of the DGN in the accessory lobe**

The DGN branches in each of the approximately 17,000 glomeruli that lie within the accessory lobe. In the electron micrographs of unlabelled preparations, the glomeruli can be distinguished from the surrounding neuropil by their darker appearance. This is a consequence both of the denser packing of very small-diameter (0.1–0.3 μm) neurites that pervade the entire volume of the glomerulus and the presence of synaptic vesicles within these neurites. In labelled preparations, irregular clusters of 0.5–0.6 μm diameter processes can be identified within glomeruli that contain numerous small granular and agranular vesicles, intermingled with very small-diameter processes and with processes of similar size containing very few vesicles (Fig. 5).

Neurobiotin-labelled branches of the DGN within each glomerulus are typically about 0.2–1.0 μm and tend to be of larger diameter where mitochondria are present. They contain numerous small agranular vesicles and a few small granular and large semilucent vesicles (Fig. 5). They are
Fig. 3. a: Horizontal section, 100-μm thick, through the olfactory and accessory lobes of the right side of a brain in which the dorsal giant neuron (DGN) was filled with neurobiotin. The cell somata is out of the plane of the section but lies to the left. The primary neurite descends from the cell body and projects to the OGT neuropil (not shown) and to all glomeruli in the olfactory and accessory lobes. The projections in the found mainly adjacent to the vesicle-containing processes from which they receive synaptic input (Fig. 5). The high density of this input was clear both from the large proportion of DGN profiles that received such synapses in any one section and by the frequency at which two or more inputs were received by a single profile. The input synapses are usually dyadic, with the DGN contributing only one of the postsynaptic elements. When sectioned tangentially, the synaptic site could be seen to be marked by a row of presynaptic densities (Fig. 5c). It was not possible to distinguish different classes of presynaptic processes. All contained predominantly small (40–50 nm) agranular vesicles accompanied by a few small (65–95 nm) granular vesicles. Neurites containing large granular vesicles are rarely encountered in the accessory lobe. The DGN was only rarely seen to make conventional output synapses with a well-developed presynaptic density (Fig. 5d).

Terminals of the DGN in the olfactory lobe

Processes of the DGN in the olfactory lobe extend through the central two-thirds of the columnar olfactory glomeruli. Unlike the accessory lobe glomeruli, the boundaries of the olfactory lobe glomeruli cannot be as clearly determined on the basis of neuropil architecture. DGN neurites labelled with neurobiotin were 0.2–0.75 μm in diameter and contained a vesicle population similar to that seen elsewhere in the neuron (Fig. 6c,d). Sometimes the labelled neurites lie next to unstained neurites with a similar vesicle content. The density of the arborisation of the DGN is much less in the olfactory lobe than in the accessory lobe. The olfactory lobe branches of the DGN also receive input synapses (Fig. 6), although at a lower frequency than accessory lobe branches. The presynaptic processes contained a vesicle compliment similar to those in the accessory lobe. Conventional output synapses were rarely observed in the olfactory lobe.

Terminals of the DGN in the olfactory globular tract

The olfactory globular tract (Fig. 7a) is comprised of a large number of tightly packed axons. Most of these are only 0.2–0.3 μm in diameter together with some larger 0.5–0.7 μm axons. Scattered among these axons, just medial to the olfactory lobes, are a number of small lens-shaped neuropils about 3–6 μm in diameter (Fig. 7b). The neurites within these neuropils are typically 0.4–0.5 μm in diameter, and the majority contain small round agranular (40–50 nm) vesicles together with a smaller proportion of small agranular (65–95 nm) vesicles. A minority of processes contain many large (150–220 nm)
Fig. 4. Sagittal sections, 100-μm thick, through the medial (a) and lateral (b) lobes of the deutocerebral neuropil (DCN) were stained with Toluidine blue. The deutocerebral commissure contains thick and thin axons, grouped into two separate bundles (DC1 and DC2). The olfactory globular tract (OGT) lies posteriorly (a) and then passes ventral to the DC (b) before branching in the olfactory and accessory lobes, which are out of the plane of these sections. The thick axons of the DC extend into the medial portion of the DCN, visible in a, and the thin axons into the lateral part of the DCN, visible in b (arrows). Scale bar = 50 μm.

granular vesicles in addition to small granular and agranular vesicles. The presynaptic structures seen in these small neuropils are generally round discrete densities of about 60 nm in diameter surrounded by clusters of vesicles. These presynaptic densities lie either in straight or curving lines (Fig. 7c).

In labelled preparations, it can be seen that the DGN sends fine neurites into many of the islands of neuropil within the OGT (Fig. 7a,b). These neurites are 0.2–0.8 μm in diameter but sometimes enlarge as varicosities 1.5 × 3 μm in diameter (Fig. 7b,e). The DGN processes contain small (50–65 nm) agranular vesicles together with some large (85–180 nm) vesicles of variable electron density (Fig. 6c). These large semilucent vesicles were particularly abundant in the varicosities (Fig. 7e). The DGN neurites receive many input synapses from the neuropilar processes containing small granular and agranular vesicles (Fig. 7c,d). Some of these inputs are clearly dyadic, whereas at other synapses the DGN appears to be the only postsynaptic element. However, because several synaptic densities often lie in a row opposite the DGN neurite at these synapses, a second postsynaptic process may lie out of the plane of section. DGN neurites in the OGT neuropil were occasionally seen to lie adjacent to
Fig. 5. Branches of DGN in the accessory lobe. a: A low power electron micrograph shows the abundance of labelled DGN terminal branches within an accessory lobe glomerulus. b: A DGN terminal branch receives four input synapses (arrowheads) in close proximity. c: A DGN terminal branch containing small agranular vesicles receives two input synapses (arrowheads). At one of these, a row of synaptic densities lies against the presynaptic membrane. d: Two lightly labelled DGN branches are visible in this micrograph (asterisks). One receives an input synapse (arrowhead) and one makes an output synapse (double arrowhead). Scale bars = 2 μm in a–c, 0.25 μm in d.
processes containing large granular vesicles but were not seen to receive conventional input synapses from them.

**Terminals of the DC interneurons in the protocerebral neuropil**

The DC neurons that were chosen for this study all responded to photic stimulation of the eyes by increasing their tonic discharge rate and hence belong to the class 2 interneurons described by Sandeman et al. (1995). This class of interneuron has unilateral branches in the median protocerebrum and DC neuropil and bilateral projections to the accessory lobes (Fig. 2).

The arborisation of such a DC neuron in the protocerebrum is composed of large diameter branches of over 1 μm from which much finer (0.3–0.4 μm) branches arise (Fig. 8a). These fine processes receive input synapses from processes containing predominantly small (30–60 nm) round agranular and granular (70–90 nm) vesicles (Fig. 8b).
SYNAPTIC TERMINALS IN CRAYFISH BRAIN

Terminals of the DC interneurons in the DC neuropil

The medial one-half of the DC neuropil, where the DC neurons labelled in this study branch (Fig. 9a,b), is comprised mainly of 0.7–1.5-μm diameter processes filled predominantly with small agranular vesicles and accompanied by a few small agranular or large semilucent vesicles. The neuropil is characterised by the prevalence of an unusual synaptic arrangement at which, in place of the normal dyadic arrangement (where the presynaptic density in the single presynaptic process lies opposite two postsynaptic elements), two or even all three of the processes involved have presynaptic bars that lie opposite one another (Fig. 9c,e,f).

The neurites of the labelled DC neurons contain mainly small agranular vesicles, with a few semilucent vesicles (Fig. 9c,e,f). They are typically 0.5–0.7 μm in diameter and are therefore among the smaller processes in the neuropil. They receive many input synapses, which may be single, of conventional dyadic structure (Fig. 9d) or of the form where two of the three processes involved contain presynaptic densities (Fig. 9c,e). The DC neuron also makes a smaller number of output synapses (Fig. 9f).

Terminals of the DC interneurons in the accessory lobe

The DC interneurons end in complex, highly branched arbors that extend through an entire accessory lobe glomerulus. The terminal branches in glomeruli measure 0.2–1 μm in diameter (Fig. 10). Processes of all diameters are generally tightly filled with small agranular and semilucent vesicles and, in contrast to the DGN, make numerous output synapses (Fig. 10c,d). These output synapses are dyadic and are typified by a distinct electron-dense thickening of the postsynaptic membranes, which extends some distance from the presynaptic dense bar. Many of the postsynaptic processes are amongst the smallest of the neurites within the glomeruli. The mean diameter of 50 postsynaptic processes was 0.23 μm.

The DC neurites also receive many input synapses close to their outputs (Fig. 10b). Occasionally, reciprocal connections appear between a DC neurite and one in the glomerular neuropil (Fig. 10d).

The distribution of DC and DGN neurites in the same glomerulus

Preparations in which both the DGN and DC neurons were labelled allowed us to examine glomeruli containing neurites from both neurons (Fig. 11). An unexpected but convenient result in such preparations was a clear difference in the nature of the label of the different neurons; the labelling of the DGN is darker and more granular in texture than that of the DC neuron (Fig. 11c,d). This difference could be confirmed by comparing the two sides of the same brain in which the DGN on only one side had been filled. The bilateral projection of the DC interneurons results in accessory lobe glomeruli on the side contralateral to the DGN containing only DC branches, whereas on the other side the glomeruli contain branches of either both neurons or the DGN alone. The processes of the DC interneurons and the DGN could also be clearly distinguished on other morphological criteria. The DC interneuron processes contain a densely packed population of synaptic vesicles and make many more output synapses, whereas the DGN processes contain loosely scattered vesicles and rarely make output synapses. For our purposes, however, it was not an absolute requirement to be able to identify the origin of each single process because our search was for synaptic contacts between any two labelled profiles in the double labelled preparations. The arbors of both the DGN and the DC interneuron extend to all parts of a glomerulus so that it is unlikely that we would see only one or the other in a section through the glomerulus.

No labelled profiles in double-labelled preparations were ever seen to form synapses with one another. Profiles that we tentatively identified as DGN and DC interneurons by the criteria described earlier sometimes lie within a micron of each other in glomeruli supplied by both neurons (Figs. 11c,d) but were never seen to come into direct apposition or make synapses with one another. DGN neurites tend to be surrounded by the larger (up to 1.0 μm diameter) vesicle-containing process, whereas the DC neurites, although approached by a few such processes from which they receive input, are more often surrounded by small (0.1–0.4 μm in diameter) neurites containing few vesicles and onto which they make their outputs.

DISCUSSION

Serotonergic neuronal terminals

Serotonergic neuronal terminals in arthropods fall into three categories. The first lie in the neural sheath (Livingstone et al., 1981; Nüssel and Elekes, 1984 and 1985), the second belong to neurons innervating the viscera (Klemm et al., 1986; Nüssel, 1988; Peters et al., 1987), where they often lie close to muscle cells or glandular tissue, and the third belong to neurons whose branches lie entirely within the central nervous system (Nüssel et al., 1985; Salecker and Distler, 1990).

Among the arthropods, the ultrastructure of terminals immunoreactive for serotonin has been most widely investigated in insects. In insects, they contain two populations of vesicles, one of agranular vesicles about 50–60 nm in diameter, the other of larger round and granular vesicles with a diameter of 80–150 nm. On the basis of preembedding immunocytochemistry, both are said to be immunoreactive for serotonin; however, as other nonvesicular structures are also labelled, this conclusion should be treated with caution. The relative proportions of small agranular and large granular vesicles can vary widely from profile to profile, raising the possibility that either several distinct classes of neurons are being labelled by the antibodies or that the distributions of the two types of vesicle within the neuron are not identical.

Neuronal terminals in the neural sheath of lobster thoracic nerves that take up radioactive serotonin have a rather different vesicle population. Although they contain some 45–70 nm round or pleomorphic agranular vesicles, the majority are larger tubular or flattened vesicles with moderately electron-dense cores (Livingstone et al., 1981). The vesicle populations within the DGN in our study were similar to these, being comprised of 50–65-nm agranular vesicles together with larger (85–180 nm) semilucent vesicles. The former were usually in the majority except in varicosities in the OGTN, which were packed almost exclusively with the latter.

Physiological studies of the DGN neuron suggest that it receives input from the accessory lobe and that action potentials are initiated here and pass to its branches in the
olfactory lobe. Output synapses were rarely seen on any of its branches, however. In insects and crustaceans, serotonin-immunoreactive terminals in the neural sheath (Livingstone et al., 1981; Nässel and Elekes, 1984 and 1985) exhibit no synaptic specialisations. Omega profiles suggestive of vesicular exocytosis are sometimes seen in terminals with morphological characteristics similar to those that are immunoreactive to serotonin (Schürmann et al., 1991) and can be seen in processes that take up 5,7-dihydroxytryptamine (Nässel and Elekes, 1985). Serotonin-immunoreactive terminals in contact with visceral muscle and within glands similarly lack synapses (Klemm et al., 1986; Nässel, 1988; Peters et al., 1987). Synapses are more regularly observed in the central nervous system, however. Serotonin-immunoreactive neurons in locust thoracic (Peters and Tyrer, 1987) and abdominal ganglia (Elekes et al., 1987) make conventional synapses as do serotonin-immunoreactive neurons in the optic lobes of the ant Cataglyphis and the bee Apis, although not in the blowfly Calliphora (Nässel et al., 1985). Synapses from serotonin-immunoreactive neurons have also been reported in the central body of the crayfish brain (Schürmann et al., 1991). Output synapses from serotonergic neurons in the olfactory lobes have been recorded both in the cockroach (Salecker and Distler, 1990) and moth (Sun et al., 1993) brains. In the latter species, however, their distribution was assessed quantitatively, and they were found to occur only at very low frequency. This reflects our findings in the crayfish DGN, although the frequency of occurrence of synapses in this neuron appears to be even lower than in the moth.

In the vertebrate nervous system, there has been an extensive and enduring debate concerning the prevalence of output synapses from serotonergic neurons (see Nieuwenhuys, 1972). Descarryes et al. (1975) concluded that only 5% of boutons of serotonergic neurons made conventional output synapses, although this was subsequently challenged by Chan-Palay (1978) and by Molliver et al. (1982) who found that synapses were present on 40–60% of serotonergic boutons in the cortex and paratrigeminal nucleus. More recent work has demonstrated a wide range in the incidence of synapses in different regions of the vertebrate central nervous system. An ultrastructural analysis of serotonin-immunoreactive neurons in the lamprey spinal cord revealed no evidence of output synapses from serially sectioned varicosities (Christensen et al., 1990), whereas similar studies in the rat spinal cord suggest that only 36% of contacts established by immunoreactive varicosities were synapt (Riedl et al., 1995). It is likely, therefore, that serotonin can be released both peripherally and centrally at nonsynaptic sites in both vertebrates and invertebrates.

**Anatomical confirmation of the physiological model for the action of DGN and DC neurons**

Physiological studies of the DGN and DC interneurons (Sandeman and Sandeman, 1994; Sandeman et al., 1995) have suggested that the DC interneurons constitute an important input to the accessory lobe and to the DGN, but that there is no direct synaptic contact between them. Instead, the results of physiological experiments suggest the interpolation of at least one set of neural elements between the DC and DGN and that these could be projection neurons. Without serial sectioning all of the glomeruli containing the branches both of a labelled DC interneuron and the labelled DGN, it is impossible to be absolutely sure that they do not make direct contact at conventional synapses; however, the results obtained do provide the following circumstantial evidence to support the hypothesis derived from the physiological recordings.

First, labelled DC interneurons have many output synapses on their bilateral projections in the accessory lobes and many input synapses on their unilateral branches in the protocerebrum and deutocerebrum, a result that justifies the conclusion that these DC interneurons carry information out to the accessory lobe.

Second, we were unable to find either intimate contact or synapses between any labelled profiles in glomeruli invaded by both DC interneuron and DGN neurites in double-labelled preparations. In a low power scan of such a glomerulus, it is possible to identify about 160 labelled profiles in each section, and most glomeruli could be positively identified in many, not necessarily serial, sections. Given the complexity of the DGN and DC arborisations and the impracticability of examining every branchlet, we cannot categorically exclude a contact between the DC interneurons and the DGN. That no contacts were ever found in scans through several levels of each glomerulus indicate, however, that any DC interneuron to DGN contact would have to be extremely rare. However, synaptic outputs from DC interneurons onto small unlabelled profiles and synaptic inputs from unlabelled processes onto the DGN were common in glomeruli containing labelled DC interneuron and DGN branches.

The physiological data has been interpreted to indicate that the DC interneurons in the accessory lobe make contact with the projection neurons, but these are not the only neurons with slender processes that end in the glomeruli of the accessory lobe. Many local interneurons also invade the glomeruli, and we have no way of knowing whether the contacts made by the DC interneurons in the accessory lobe are with the projection neurons or with the local interneurons, or both.

The electron micrographs of double filled preparations are interesting in revealing how many unlabelled profiles can be seen in thin sections of glomeruli. One possible explanation of this is that not all branches of the DC interneurons and DGN were filled; however, the evenness of the staining seen in the light and electron microscope and the similarity in the extent of the branching seen using a variety of intracellular stains make this appear unlikely. It is more probable that we are seeing the real extent of the invasion of each glomerulus by the projection and local interneurons and that they make up the greater part of the glomerular neural volume.

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**Fig. 7.** Branches of the DGN in the olfactory globular tract (OGT). a: A low power electron micrograph shows regions of neuropil in the OGT that contain DGN branches (arrows) separated by the axon bundles of the tract. b: In the light microscope, branches of the DGN can be seen in this 100-μm slice through the OGT and in the adjacent accessory lobe (AL). Some of the branches in the OGT bear large varicosities (arrows). The electron micrographs were taken from ultrathin sections cut from this slice. c: A lightly labelled DGN branch (asterisk) receives an input synapse (arrowhead) at which a row of synaptic densities lie along the presynaptic membrane. Note also the presence of a twisting line of presynaptic densities (arrows) in a nearby unlabelled process. d: A DGN terminal branch (asterisk) in the OGT neuropil containing some small agranular and large semilucent vesicles receives an input synapse (arrowhead). e: A large varicosity on a DGN neurite contains many large granular vesicles of differing electron densities. Scale bars = 1 μm in a, 25 μm in b, 0.5 μm in c–e.
Fig. 8. Terminals of a DC interneuron in the protocerebral neuropil. 

**a:** A light micrograph montage of two planes of focus through a 100-μm slice shows the typical form of protocerebral branches of this neuron: a small number of short thick branches give off much finer ones that extend a short distance into the neuropil. 

**b:** Ultrathin sections cut from the same slice reveal input synapses (arrowheads) onto the fine DC interneuron branches (asterisks). Scale bars = 25 μm in a, 0.5 μm in b.

The present view of the role of the DGN is that it may modulate the processing of olfactory input within the olfactory lobe. We did not find many convincing examples of conventional synaptic outputs from the DGN in the olfactory lobe to support this, however; neither did we find many output synapses from this neuron in the accessory lobe or in the OGT neuropil. The difficulties in making assumptions about the release of serotonin from neurons on anatomical evidence has already been discussed. An interpretation that may be placed on this result is that the DGN can release serotonin in both accessory lobe and olfactory lobe.

The OGT neuropil was first observed by Helm (1928) and later described by Tsvilineva and Titova (1985). It is more diffuse than most central neuropils and unusual in consisting of small islands of synaptic processes distributed among the axons of the OGT. That branches of the DGN ramify among the axons of the OGT was first reported following labelling of the brain with antibodies against serotonin (Sandeman and Sandeman, 1987) and was later confirmed by intracellular injection of the DGN with neurobiotin. If the input synapses onto the DGN that we describe here are from the projection neurons in the OGT, this would account for the excitation of the DGN when the OGT was electrically stimulated (Sandeman and Sandeman, 1994). The DGN, therefore, has inputs both in the accessory lobe (via the DC) and in the OGT (via the projection neurons), a feature that is consistent with the physiological results. The DGN is, however, not the only neuron with branches into the OGT, and the input synapses we see have still to be shown to be from the projection neurons.

**Synaptic configurations in the DC neuropil**

The DC neuropil, variously called the “lateral glomeruli” or “parolfactory lobes” (see Sandeman et al., 1992 for a review of the nomenclature) have been identified in only those decapods with accessory lobes. All the 1,000 small and 400 large axon neurons in the deutocerebral commissure of the crayfish appear to have projections to this neuropil and in silver-impregnated preparations; axons from the lateral protocerebrum also appear to enter it. In spite of this massive convergence on a fairly small neuropil, single-labelled DC neurons branch profusely within either its medial or lateral one-half and appear to contribute a significant proportion of the total volume. The synaptic configurations in the DC neuropil are unusual and could provide a clue to its function.

At the typical dyadic synapse seen in the arthropod nervous system, a single presynaptic process makes contact with two postsynaptic processes. The DC neuropil, however, is characterised by a very high incidence of a more complex synaptic configuration. This also comprises three neuronal elements, but where their membranes come together, two or even all three exhibit presynaptic dense bars. Similar configurations have occasionally been reported elsewhere in the arthropod nervous system. For example, at rare output synapses made by prosternal hair afferent neurons in the locust, both the afferent and a second element have presynaptic bars (Watson and Pfüger, 1984). The most notable case, however, is found at synapses made between the fast extensor tibiae (FETi) motor neuron and flexor motor neurons in the locust metathoracic ganglion (Burrows et al., 1989). Here the circuitry in which the synapse acts is partially understood, and a possible rationale for the synaptic arrangement can be suggested. The flexor and extensor motor neurons are active together when the muscles they supply cocontract as part of the motor program for the jump. This coactivation is brought about by common driving from a third neuron (Pearson and Robertson 1981) and is reinforced by the connection between the motorneurons. At most of the synapses from the FETi onto flexor motorneurons, a third unidentified element that also contains a presynaptic bar is also present (Burrows et al., 1989). One hypothesis to explain this arrangement is that the unidentified element belongs to the neuron that provides the common drive to the FETi and flexor.
Fig. 9. Terminals of a DC interneuron in the DC neuropil. a: A light micrograph from a 100-μm slice shows the structure of the DC neuron's branches in the medial part of the DC neuropil. The electron micrographs come from the same slice. b: A low power electron micrograph shows the abundance of the labelled branches from this single neuron within the neuropil. c: A vesicle-containing DC branch receives two adjacent inputs (arrowheads) at the same synapse. d: A small DC process receives two input synapses (arrowheads) at separate synapses. e: A DC branch receives two inputs at one synapse and a third at a second synapse (arrowheads). f: A DC branch receives an input synapse (arrowhead) and makes an output synapse (double arrowhead). Nearby is a synaptic association (circle) where all three unlabelled processes involved contain presynaptic densities. Scale bars = 25 μm in a 1 μm in b, 0.5 μm in c-f.
In the DC neuropil, the appearance and content of the labelled neurites of the labelled DC interneurons resemble those contributing to the synaptic complexes. Occasionally, the labelled neurite forms one element of such a complex and both receives and makes synapses. The DC neuropil may therefore be a region where different DC neurons interact with each other. The branches of the DC neuron examined are amongst those of the smallest diameter within the neuropil. The terminal arbours of DC neurons within this neuropil can be divided into a number of categories on morphological grounds. Many of the others are comprised of much coarser branches.

Where input and output synapses are intermingled on a single neurite (Watson and Burrows, 1988) as they are in the DC neuropil, it is possible that synapses on the postsynaptic neuron may be activated by subthreshold potentials. There is evidence that some spiking crustacean neurons can release transmitter in the absence of action potentials (Graubard et al., 1983; Nagayama et al., 1983). Another possibility is that these synaptic complexes are sites where neurons can influence each other's output by presynaptic inhibition or facilitation.

The DC neuropil is shared by all the DC interneurons that supply the accessory lobe and is synaptically very complex. Although clearly an important component for the input side of the DC interneuron/accessory lobe system, it requires further physiological and anatomical investigation before its significance can be made clear.

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Figure 11
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