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Tyrosine hydroxylase in the cerebral ganglia of the American cockroach (*Periplaneta americana* L.): an immunohistochemical study

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Abstract. We have investigated the distribution of tyrosine-hydroxylase-like immunoreactivity in the cerebral ganglia of the American cockroach, *Periplaneta americana*. Groups of tyrosine-hydroxylase-immunoreactive cell bodies occur in various parts of the three regions of the cerebral ganglia. In the protocerebrum, single large neurons or small groups of neurons are located in the lateral neuropil, adjacent to the calyces, and in the dorsal portion of the pars intercerebralis. Small scattered cell bodies are found in the outer layers of the optic lobe, and clusters of larger cell bodies can be found in the deutocerebrum, medial and lateral to the antennal glomeruli. Thick bundles of tyrosine-hydroxylase-positive nerve fibers traverse the neuropil in the proto- and deutocerebrum and innervate the glomerular and the nonglomerular neuropil with fine varicose terminals. Dense terminal patterns are present in the medulla and lobula of the optic lobe, the pars intercerebralis, the medial tritocerebrum, and the area surrounding the antennal glomeruli, the central body and the mushroom bodies. The pattern of tyrosine-hydroxylase-like immunoreactivity is similar to that previously described for catecholaminergic neurons, but it is distinctly different from the distribution of histaminergic and serotonergic neurons.

Key words: Tyrosine hydroxylase – Catecholamine neurons – Invertebrate nervous system – Immunohistochemistry – Cerebral ganglia – *Periplaneta americana* (Insecta)

Introduction

Insect brains are ideally suited for studies of neuronal circuitry. The presence of large, easily identifiable neurons in a small brain that can be serially sectioned to allow the reconstruction of pathways are features that make insect brains an important neuronal model system. The brain, or supraesophageal ganglion, of insects is a small, but complex, aggregation of glomerular and nonglomerular neuropil, connected by ascending and descending pathways (see Nässel 1987a; Malun et al. 1993) and is formed by the fusion of the three most anterior segmental ganglia (for a review, see Nässel 1987a). A number of neuroactive substances have been identified in insect brains. For example, a number of efferent neurons and interneurons have been shown to contain gamma aminobutyric acid (GABA; Distier 1990), gastrin-CCK (Tamarelle et al. 1990), serotonin, acetylcholine, octopamine (see Klemm 1976; Nässel 1987a; Salecker and Distier 1990), and histamine (Pirvola et al. 1988).

Tyrosine hydroxylase (TH) is the first, and rate limiting, enzyme in the synthesis of L-DOPA (3,4-dioxyphenylalanine) from tyrosine in mammals (Nagatsu et al. 1964). Dopamine and noradrenaline have long been known to be present in cockroach cerebral ganglia (Frontali and Norberg 1966; Frontali and Häggendal 1969), and catecholaminergic neurons have been identified by aldehyde-fluorescence techniques in the protocerebrum (PC) and deutocerebrum (DC) of the brain of the American cockroach (Frontali and Norberg 1966; Elofsson and Klemm 1972; Klemm 1983). These studies have recently been confirmed by dopamine immunohistochemistry, showing a dense plexus of dopamine-immunoreactive cell bodies and fiber terminals in the cockroach brain (Milton et al. 1991). However, the involvement of TH in catecholamine synthesis in insects has been questioned (Vaughan and Neuhoff 1976; Mir and Vaughan 1981). There is strong evidence for the presence of TH in the cerebral ganglia of dipterans (Budnik and White 1987, 1988; Neckamayer and Quinn 1989). However, dipteran brains, like those of some crustaceans (Elofsson and Klemm 1972), have unusually high levels of L-DOPA (Nässel and Laxmyr 1983) compared with the relatively low L-DOPA levels in cockroach cerebral ganglia (Owen and Bouquillon 1992). Experiments showing that L-DOPA synthesis in isolated *Periplaneta americana* cerebral ganglia is (1) blocked by the TH inhibitor alpha-methyl tyrosine and (2) stimulated by the addition of L-tyrosine strongly suggest the involvement

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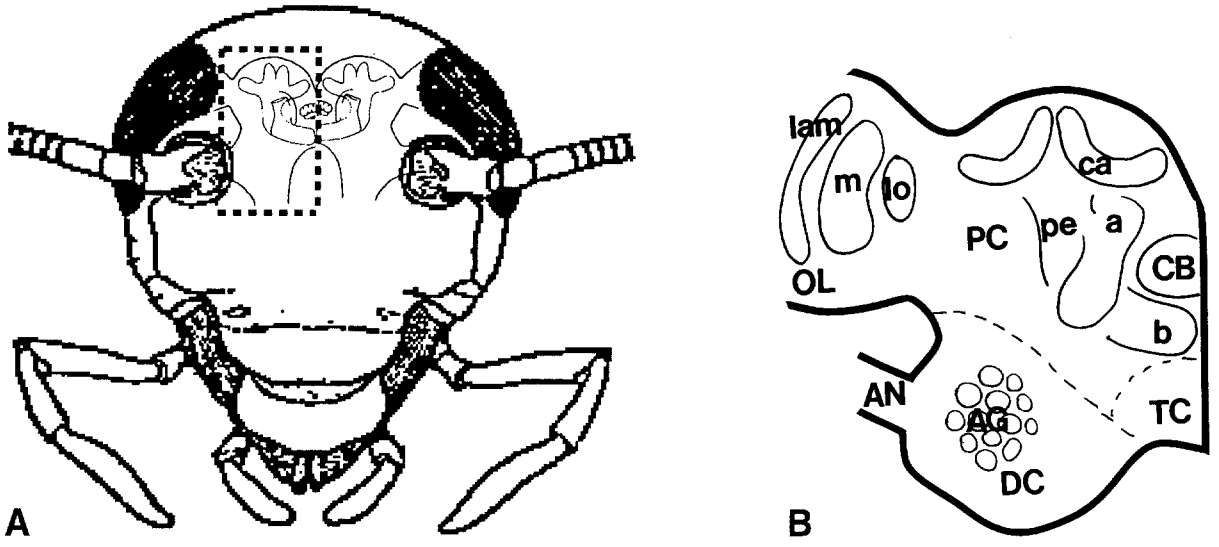

Fig. 1. **A** Frontal view of the head of *Periplaneta* showing the position of the central nervous system. The *dotted square* is an approximate indication of the area shown in **B**. Composite of diagrams modified from Pipa and Delcomyn (1982), and Milton et al. (1991). The abbreviations, which are used throughout the figures and legends, are as follows: *a* alpha lobe of mushroom bodies (MB); *AG* antennal glomeruli; *AN* antennal nerve; *b* beta lobe of MB; *ca* calyces of MB; *CB* central body; *DC* deutocerebrum; *lam* lamina of optic lobe; *lo* lobula or lobular plate of optic lobe; *m* medulla of optic lobe; *OL* optic lobe; *PC* protocerebrum; *pe* peduncles of MB; *TC* tritocerebrum

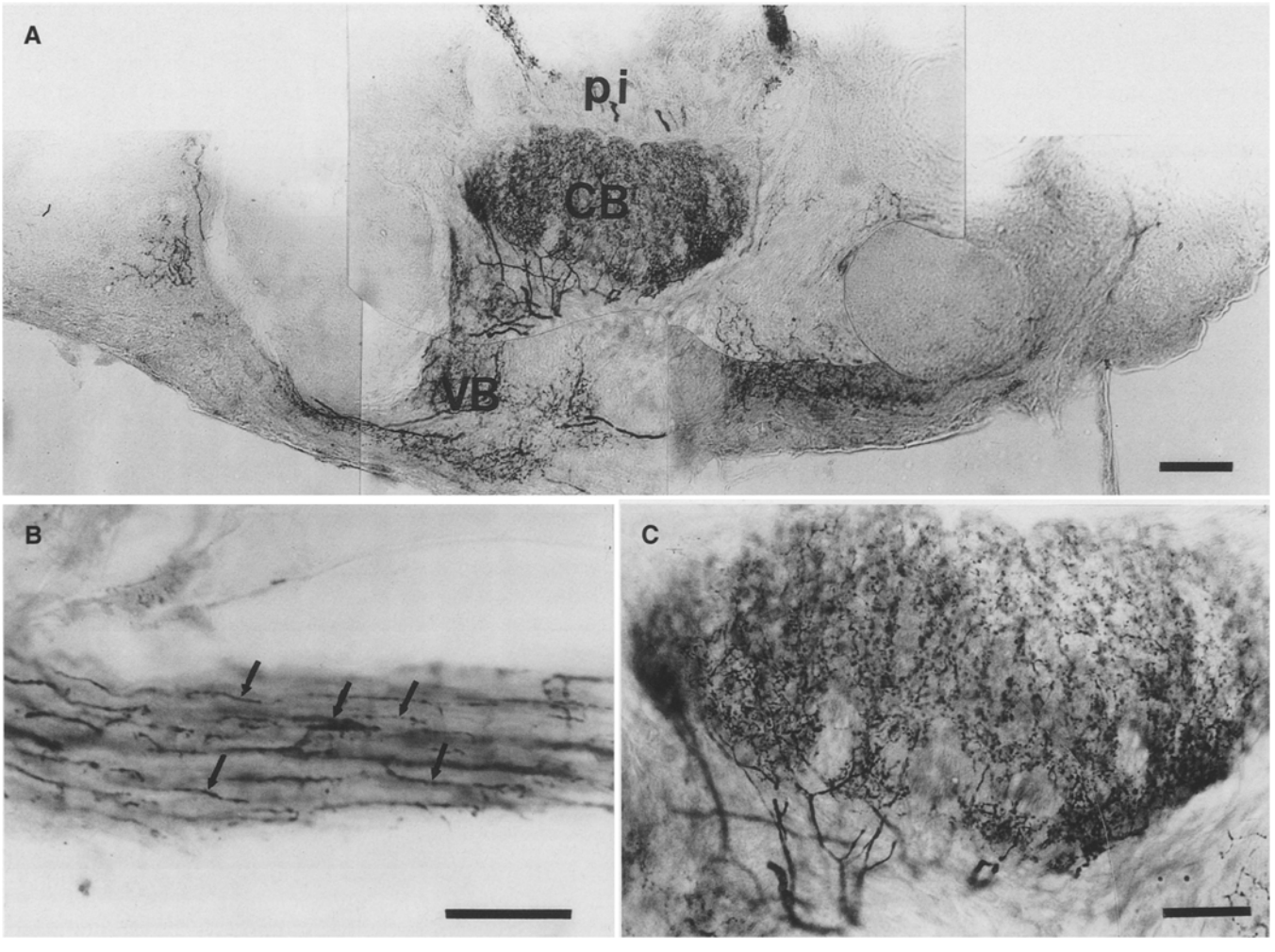

Fig. 2. **A** Photomontage from a section incubated with serotonin antibodies. Observe the dense innervation of varicose fibers in the central body (*CB*), with thick nervous trunks leading into it. *pi* Pars intercerebralis; *VB* Ventral base. **B** Thick axon bundle in the antennal nerve, containing numerous serotonin-immunoreactive nerve fibers (*arrows*). **C** Higher magnification micrograph showing the serotonergic innervation of the CB depicted in **A**. Note the dense plexus of varicose fibers, terminating throughout the CB and the thick axons leading into the CB. *Bars*: 90 μm in **A**, 50 μm in **B**, 40 μm in **C**

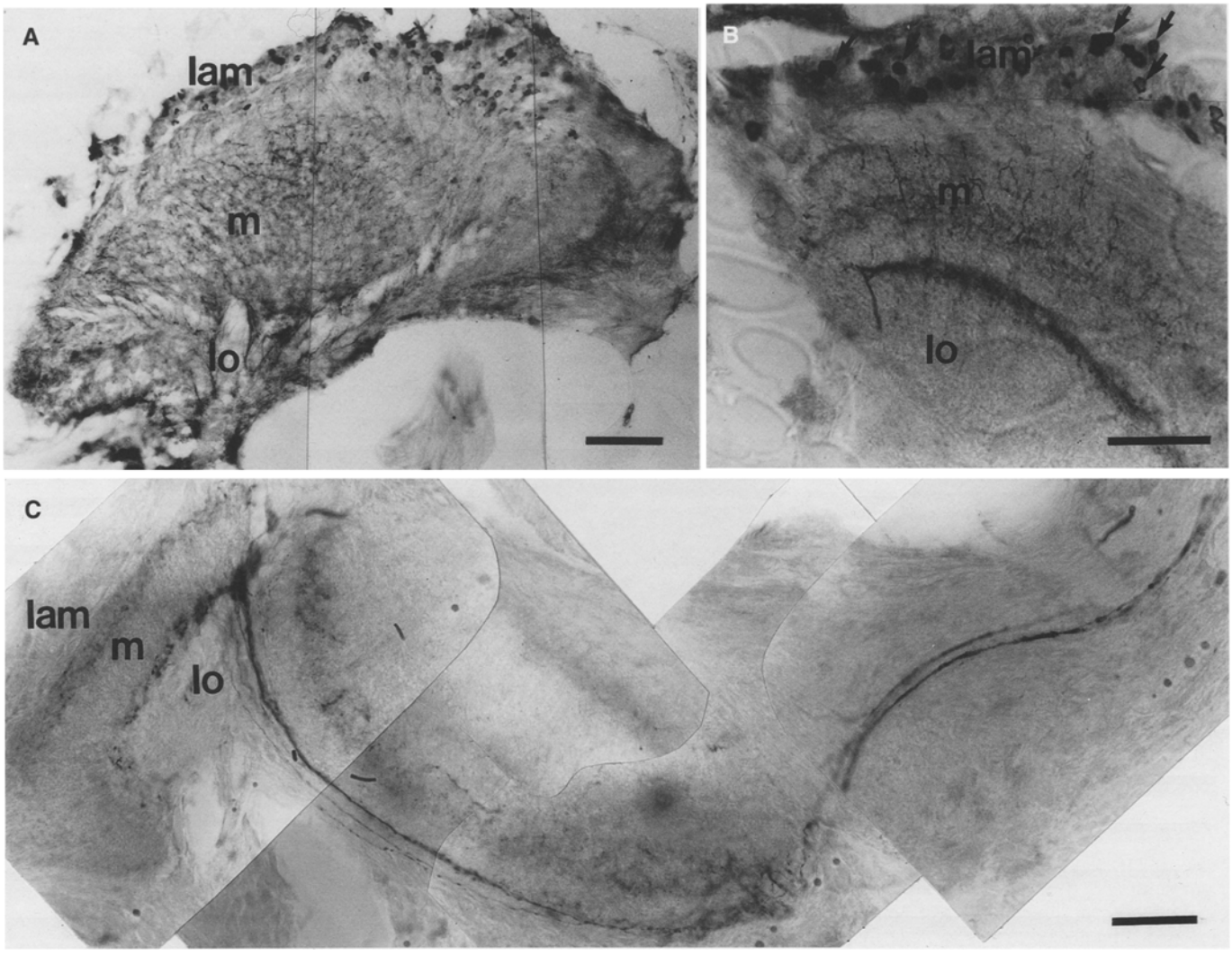

Fig. 3A–C. TH and serotonin immunoreactivity in the optic lobes of the American cockroach. **A, B** Distribution of TH-positive neuron cell bodies (*small arrows*) and nerve fiber plexus in the lamina (*lam*) and medulla (*m*). **C** Distribution of serotonin-positive nerve fibers innervating the medulla (*m*). Note that there are no serotonin-immunoreactive cell bodies in the medulla, although numerous TH-positive cell bodies are present in this region. *lo* Lobula. *Bars*: 50 μm in **A, B**, 40 μm in **C**

of TH in cockroach L-DOPA synthesis. However, only negative results have come from attempts to demonstrate TH in cockroach ganglia by radioenzymatic or LCEC assays (Owen and Bouquillon 1992).

Immunohistochemistry, which demonstrates and maps TH immunoreactivity in the cockroach brain, offers a fresh approach to the problem. If TH immunoreactivity can be localized in the same regions as those previously shown to contain catecholamines, then this provides strong evidence that TH is indeed involved in the synthesis of L-DOPA from tyrosine in neurons within the cerebral ganglia of the cockroach.

Materials and methods

Cockroaches (*Periplaneta americana L.*) were chilled to immobility, ligatured behind the head, which was then cut off, and the ganglia were removed under cold fixative, viz., 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), and fixed overnight in fresh fixative. Fixed ganglia were transferred to 30% sucrose in PBS, and embedded in 10% gelatin in plastic capsules that were allowed to solidify for 15–30 min in a vacuum before the plastic capsules were removed. Gelatin blocks were fixed in 4% paraformaldehyde in PBS overnight and transferred to 30% sucrose in PBS for at least 24 h. Vibratome sections (30 μm thick) were collected from each capsule. Sections for routine histology were mounted on chrome-alum-coated slides, stained with toluidine blue, dehydrated, mounted in DPX neutral mounting medium, and examined by light microscopy. For immunohistochemistry, the sections were placed in dilution medium (PBS+0.05% Triton X-100) in tissue trays. The sections were rinsed 3×10 min in dilution medium followed by 15 min in 3% H_2O_2 in dilution medium to inhibit residual endogenous peroxide. [All washes and incubations were done on a shaker table.] The sections were rinsed 3×10 min in dilution medium, followed by a 60-min incubation in 3% normal goat serum (NGS) and 2% bovine serum albumin (BSA) in dilution medium to block background staining. Antibodies were diluted in PBS with 1% NGS, 1% BSA, 0.3% Triton X-100, and sections were incubated for 48–72 h at 4°C. The antibodies used in this study were directed

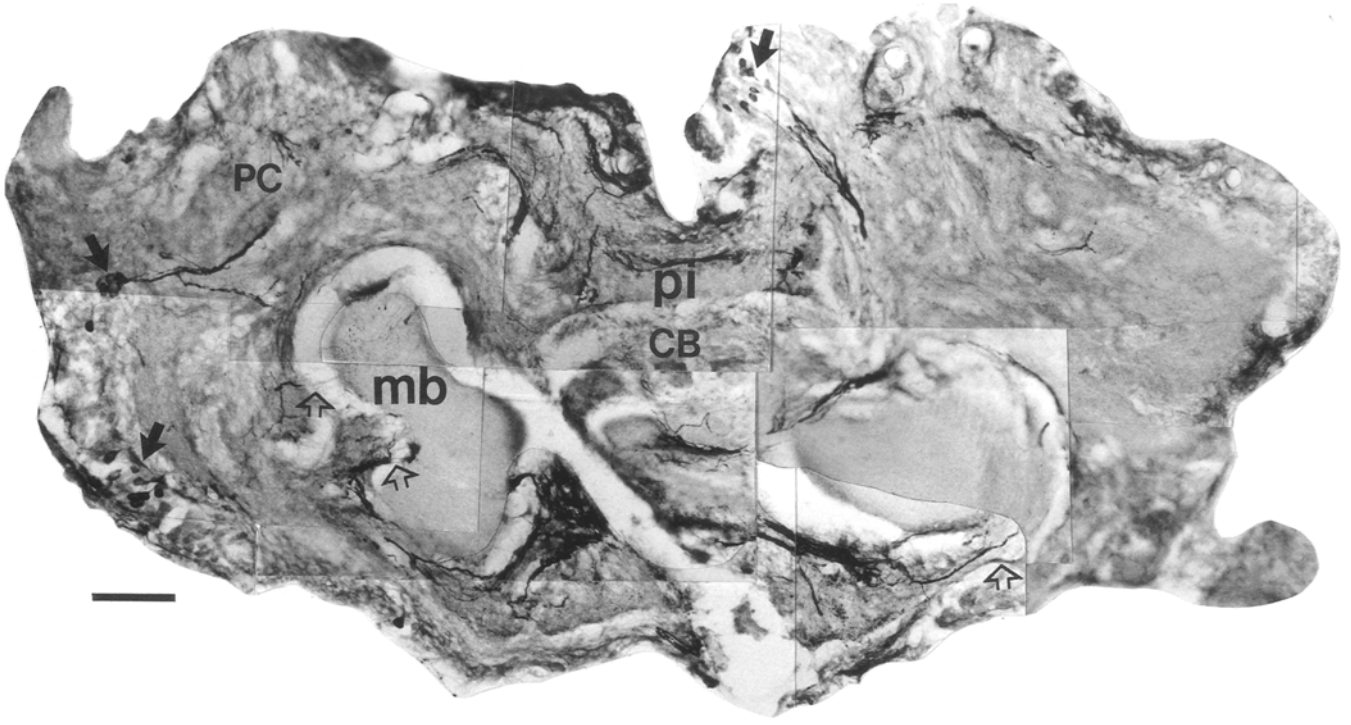

Fig. 4. Photomontage illustrating a frontal section through the ventral ganglia. Numerous clusters of TH-positive cell bodies can be seen in the lateral and medial portions of the nonglomerular protocerebral neuropil (*PC*) (see *filled arrows*). The cell bodies have thick axons converging into axonal tracts toward the midline. Fibers crossing to the contralateral side are seen in the pars intercerebralis (*pi*) above and below the central body (*CB*). The glomerular tissues are innervated by TH-immunoreactive fibers that enter the glomerular tissues at the points shown by *open arrows*. *mb* Mushroom bodies. *Bar*: 75 μm

against TH (Eugene Tech, Allendale, N.J., USA; dilution 1:1000) and serotonin (Dako Corp., Carpinteria, Calif., USA; dilution 1:1000). When "immunoreactive" or "positive" is mentioned in the text, this always refers to "like-immunoreactive", based on the specificity tests earlier performed for these antibodies (see Granholm 1991). After the incubation, the sections were rinsed 3×10 min in dilution medium, followed by 1 h in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, Calif., USA) in 1% NGS, 1% BSA in dilution medium. The sections were rinsed 3×10 min in dilution medium and then incubated with the avidin-biotin complex (ABC Elite Substrate; Vector Labs), in the same solution as that used for the biotinylated secondary antibody, for 75 min. Sections were then rinsed 3×10 min in PBS before being developed for 5–15 min with 0.05% diaminobenzidine, 0.3 ppm nickel ammonium sulfate, 0.1 ppm H_2O_2 in PBS. Sections were then rinsed 4×10 min in PBS, mounted and dehydrated on chrome-alum-coated glass slides and overslipped with DPX neutral mounting medium and analyzed by light microscopy. Sections where the first and the second antibodies had been omitted served as controls for each sectioned brain. TH-immunoreactive cell bodies were measured using microscope calibration bars in a Nikon Optiphot microscope coupled via a Cohu video camera (model 4990) to a Scion Frame Grabber Card in a Macintosh Quadra 840V computer with the NIH Image analysis system. The calibration bars allowed for accurate measurement of cell body areas on a captured image.

Results

Morphology of cockroach brain

The schematic drawing in Fig. 1 shows the location (Fig. 1A) and the regional anatomy (Fig. 1B) of the brain of the American cockroach (*P. americana*). This is included to simplify the interpretations of the immunohistochemical data. The cockroach supraesophageal ganglion has three subdivisions: (1) the DC with the antennal lobe (AG), (2) the PC with the mushroom bodies (MB) and the central body complex (CB), and (3) the tritocerebrum (TC) (Fig. 1B). The optic lobes (OL) have been suggested to be part of the deutocerebrum (Pipa and Delcomyn 1982).

Immunohistochemical observations

In order to demonstrate that results obtained with the ABC immunotechnique are comparable with the previous aldehyde-fluorescence studies, the distribution of serotonin-like immunoreactivity in the cockroach brain was examined. The presence of characteristic serotonin-like innervation patterns in the CB in the midline of the PC is shown in Fig. 2A and C. The large number of serotonin-immunoreactive nerve fibers in the antennal nerve is shown in Fig. 2B). The pattern of serotonin innervation closely resembles that previously described by other authors (see Nässel 1987a for a review). The pattern of serotonergic innervation of the OL is illustrated in Fig. 3C. A bundle of serotonin-immunoreactive nerve fibers is seen in the lobular plate, spreading into a fine network of varicose terminals throughout the medullary plate (Fig. 3C). The TH immunoreactivity in the OL is

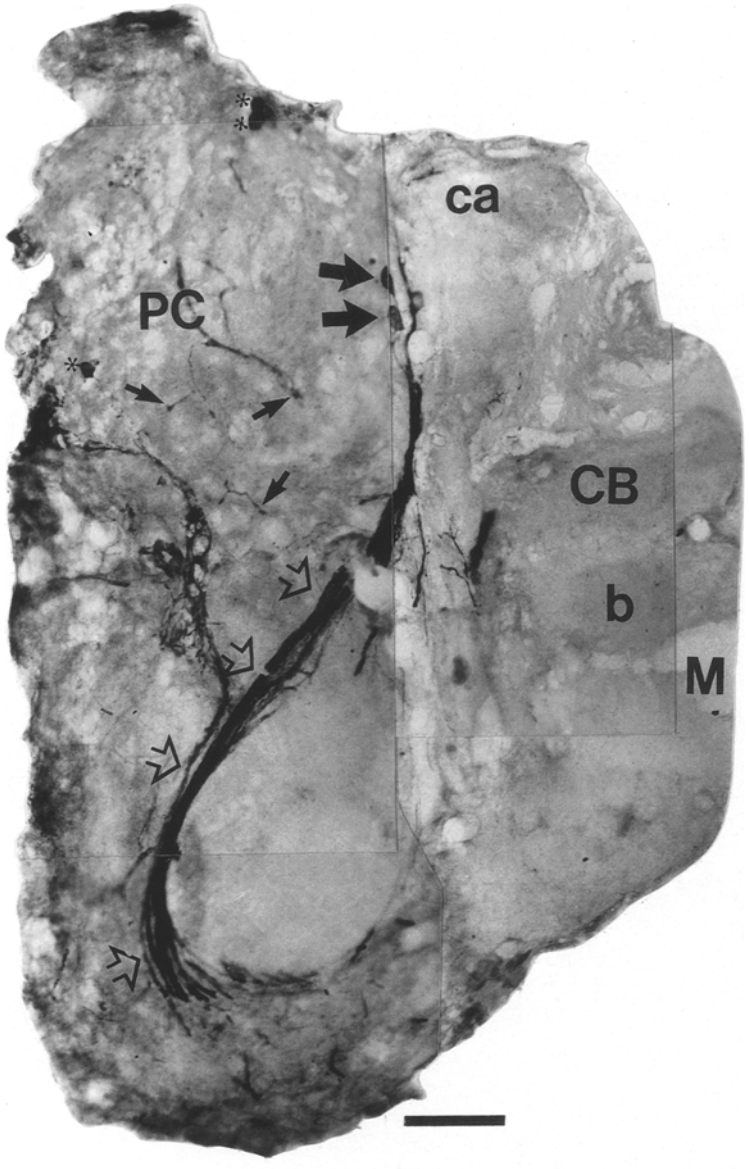

Fig. 5. Photomontage illustrating a thick tract of TH-immunoreactive fibers (*open arrows*) originating in cell clusters (*large filled arrows*) in the dorsolateral protocerebral neuropil (*PC*). TH-immunoreactive cell bodies are also located in smaller groups at the edge of the *PC*, both laterally and dorsally (*asterisks*). TH-immunoreactive varicose fibers innervate the dorsolateral *PC* (*small filled arrows*), and the axonal trunk passes through the *PC* centrally, and circumvents the peduncle of the mushroom body (*open arrows*). The central body complex (*CB*) and the betalobe (*b*) of the mushroom body are located in the midline (*M*). *ca* Calyx of the mushroom bodies. *Bar*: 80 µm

distinctly different from the serotonin immunoreactivity described above. A large number of small TH-positive cell bodies with a mean cell body diameter of 7 µm occur in the external layer of the medulla and in the lamina (Fig. 3A, B). Further, there is a dense plexus of terminals associated with these neurons in the internal medulla and in the lobula (Fig. 3A, B). Axons from these neurons leave the OL and extend toward the MB in the PC. In the PC, clusters of TH-positive cell bodies are located both in the lateral neuropil (Figs. 4, 5) and in the medial portion of the pars intercerebralis (Fig. 6). The mean diameter of these TH-immunoreactive protocerebral cell bodies is 15 µm for the lateral cells, and 12 µm for the medial cell groups (Fig. 6). Thick fiber bundles traverse the PC to the contralateral side on both sides of the CB (Fig. 4). The CB and the MB are densely innervated by fine varicose terminals. One particularly thick TH-immunoreactive pathway originates in the lateral PC from several clusters of cells (Fig. 5) and traverses the non-glomerular neuropil, lateral to the peduncle of the MB where it turns toward the midline and ends in a fanlike fashion close to the border of the DC. The AG also contains clusters of cell bodies, or single cell bodies, located both laterally and medially to the antennal glomeruli (Figs. 6, 7). Axon bundles from these cell bodies innervate the antennal glomeruli (with varicose fibers) and the MB of the PC (Fig. 7). A large number of TH-immunoreactive nerve fibers also traverse the DC toward the midline of the TC where they appear to cross the midline toward the contralateral side (Fig. 7).

Discussion

The distribution of catecholaminergic neurons has been investigated in a number of different insects (Frontali 1968; Björklund and Stenevi 1970; Klemm 1976; Budnik and White 1988; Salecker and Distier 1990). Budnik

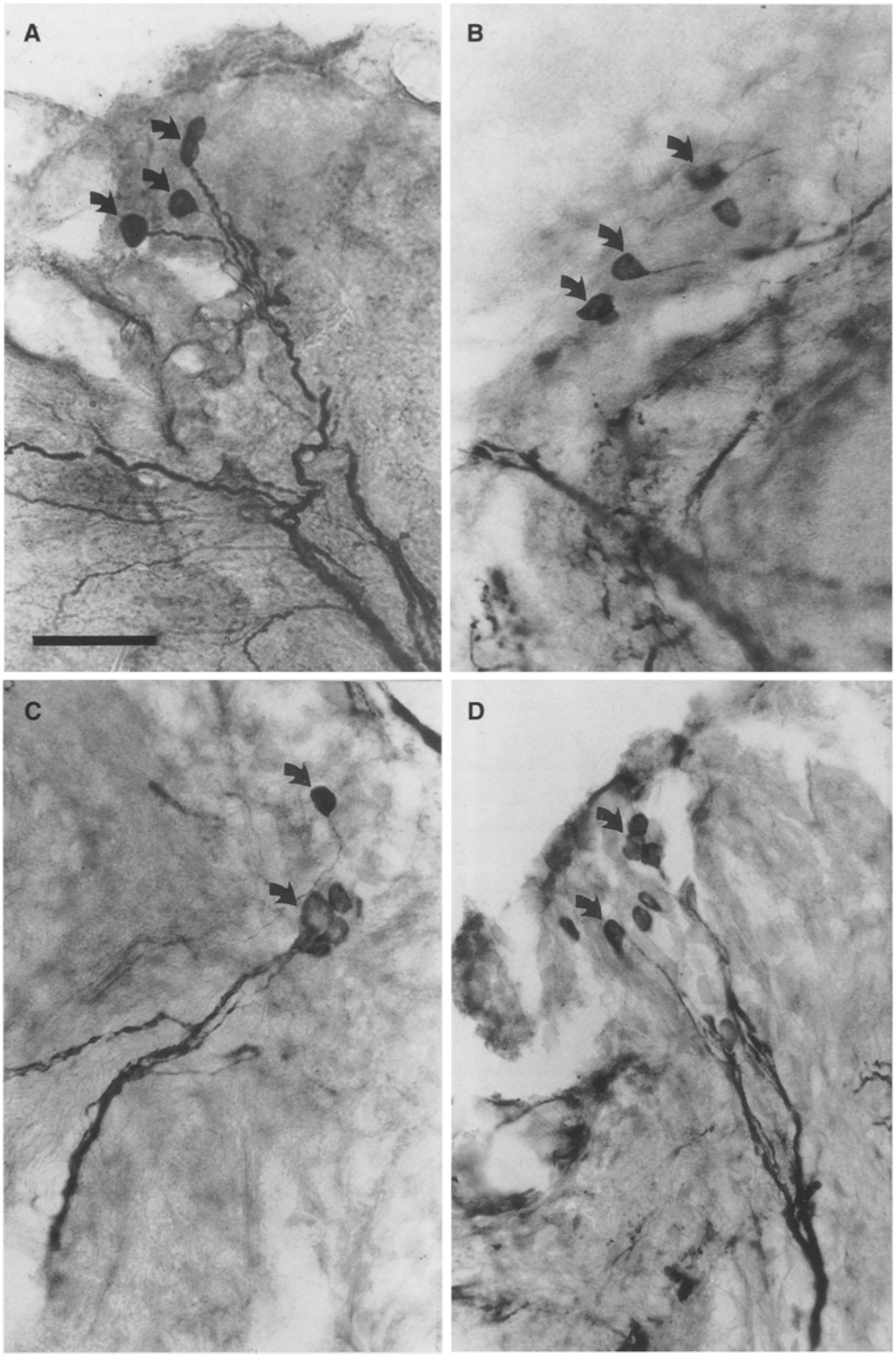

Fig. 6A–D. Higher magnification of TH-immunoreactive nerve cell bodies in the protocerebrum (**A, C, D**) and deutocerebrum (**B**). The cell bodies, which are 10–20 μm in diameter, appear to be pseudo-unipolar, with one process, a large perikaryon, and a large nucleus. The axons are mostly thick and smooth, with few varicosities. *Bar* (shown in **A**): 60 μm

and White (1988) have examined the distribution of TH and dopamine immunoreactivity in larval and adult *Drosophila melanogaster*. They have found the patterns of reaction to TH and dopamine antibody to be similar and conclude that in *Drosophila*, TH is located in the dopaminergic neurons. Our results suggest that this is also the case in the cockroach brain, since the pattern of immunoreactivity that we show with TH antibodies is similar to the histochemical localization of catecholaminergic neurons with aldehyde-fluorescence (Frontali and Norberg 1966; Klemm 1983; Nässell 1987a) and with antibodies directed against dopamine (Milton et al. 1991).

The OL of insects are organized into three main neuropil regions; the lamina (distal), the medulla, and the lobula complex (proximal) (see our Fig. 1; Nässel 1987c). The neurons of the lamina are organized in a

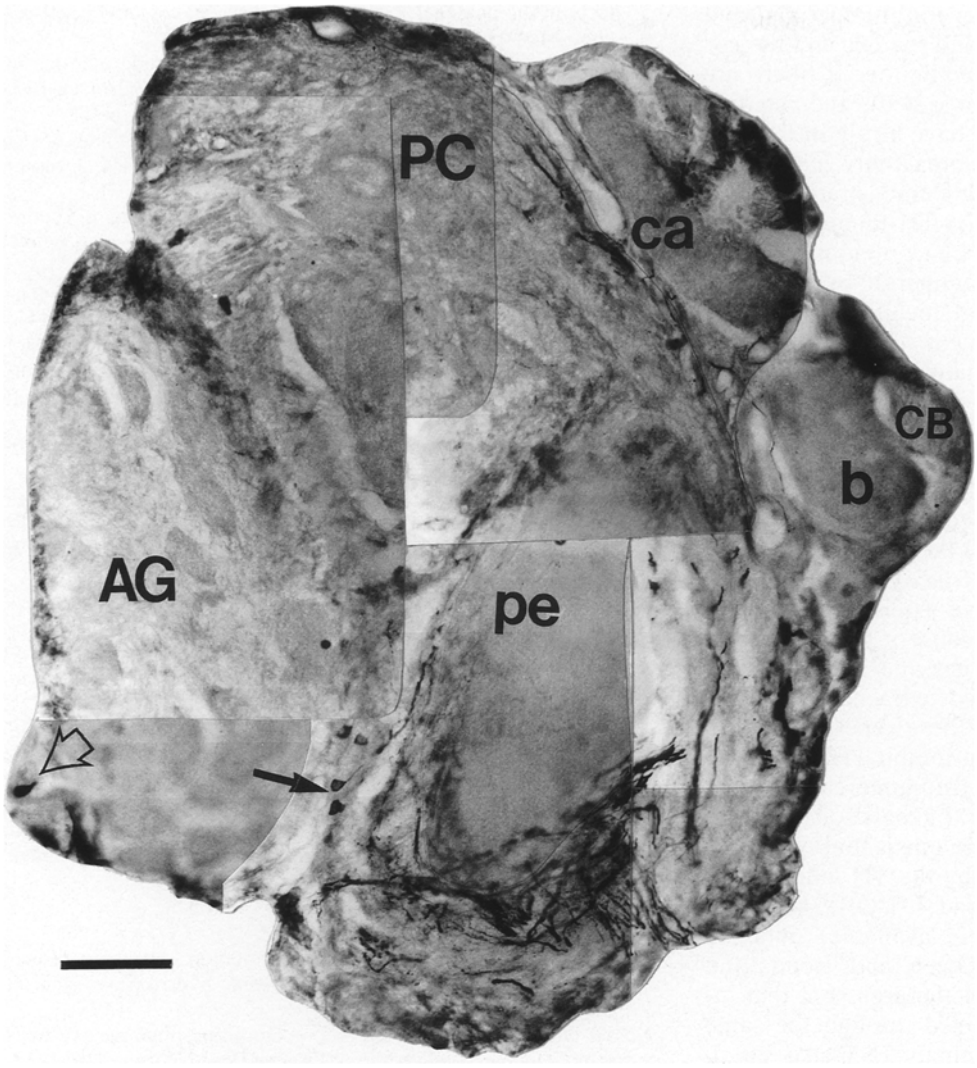

Fig. 7. Photomontage demonstrating TH immunoreactivity at the level of the antennal glomeruli (*AG*). Groups of TH-immunoreactive cell bodies are located both medial (*filled arrow*) and lateral (*open arrow*) to the *AG*. The mean diameter of these cell bodies is 15 μm. Bundles of nerve fibers surround and innervate the *AG*, the central body complex (*CB*), and the calyx (*ca*), and beta lobe (*b*) of the mushroom bodies. The TH-immunoreactive axon bundles are particularly abundant just ventral of the peduncles (below *pe*). *Bar*: 90 μm

columnar pattern that projects into the more central medulla and lobula neuropils. Ten types of neurons have been described connecting the lamina to the medulla in the insect OL. In addition, one type of intrinsic amacrine neuron and a serotonin-immunoreactive neuronal type connect the lamina, medulla, and lobula complex to the midbrain and contralateral OL (Nässel et al. 1985, 1987; Nässel 1987c). Immunoreactivity to catecholamine, serotonin, gastrin and somatostatin has been found in the laminar neurons of insects (Nässel et al. 1985, 1987). If tyrosine hydroxylation is the first step in the synthesis of insect neuronal catecholamines, then our results, showing a distinct population of TH-immunoreactive neurons in the lamina of the OL of the American cockroach, may represent the same neurons as those previously demonstrated to be catecholamine-immunoreactive. The TH-immunoreactive neurons in the lamina connect to the medulla through an intricate network of neuronal processes. There are also thick TH-immunoreactive axons that emerge from the medulla and connect to the lobula complex. We differ from previous investigators (Nässel et al. 1987) in not finding any serotonin-immunoreactive neurons in the lamina. Rather, we see a large number of serotonin-immunoreactive axons diverging into the medulla and forming a plexus that innervates medullary interneurons. It is possible that these serotonergic projections arise from cell bodies in the lobula plate, since serotonergic neurons in this plate have been shown to project to the more lateral optic neuropils (see Nässel et al. 1985; Nässel 1987b).

The CB has been shown to react to a number of different neuronal markers, including serotonin, gastrin/CCK, GABA, substance P, proctoline, and histamine (Nässel 1987b; Nässel et al. 1987; Nässel and O'Shea 1987; Pirvola et al. 1988). Catecholamine histochemistry has demonstrated the presence of a large number of catecholaminergic terminals in the CB of *Calliphora* (Klemm 1976) and in *Periplaneta* (Frontali 1968; Sloley and Owen 1982). The CB of *Periplaneta* exhibits, in our investigation, a pattern of TH-immunoreactive profiles that closely resembles the pattern of catecholamine histofluorescence presented in earlier studies.

The distribution of TH immunoreactivity that we describe shows catecholaminergic neurons to be part of an

extensive system connecting several glomerular and nonglomerular neuropil regions and the OL and the antennal glomeruli. TH-immunoreactive nerve fibers are also present in descending neuron systems and antennal nerves, suggesting that they also have a role in the sensory afferent system. TH immunoreactivity has not, to our knowledge, been previously demonstrated in the cockroach brain. The presence of TH-immunoreactive neurites and cell bodies in the same regions as those previously identified as catecholaminergic by histofluorescent and immunocytochemical techniques provides strong evidence for the involvement of TH in the synthesis of dopamine and noradrenaline from tyrosine. A proposed alternative pathway of catecholamine synthesis in insects in which tyrosine is first decarboxylated to tyramine that is then hydroxylated to produce dopamine (Vaughan and Neuhoff 1976; Mir and Vaughan 1981) has never been confirmed. Radioenzymatic (Waymire et al. 1971) and LCEC (Blank and Pike 1976) assays may have failed to demonstrate TH activity (Owen and Bouquillon 1992) either because cockroach TH demands a different cofactor or because it has a different optimal pH than the mammalian enzyme. A more likely explanation is that the unpurified extracts of cerebral ganglion tissue that were used in these experiments also contained a factor, or factors, that inhibit TH. When the results of our study, showing TH-immunoreactive neurites and cell bodies in the cerebral ganglia of *Periplaneta*, are combined with the circumstantial evidence (blockage of L-DOPA synthesis by the TH inhibitor alpha-methyl tyrosine and increased L-DOPA synthesis when more tyrosine precursor is available) obtained with biochemical techniques (Owen and Bouquillon 1992), there is strong support for the argument that insect catecholamines are synthesized through the same pathway as that found in mammals (Nagatsu et al. 1964).

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