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Identification of distinct tyraminergetic and octopaminergic neurons innervating the central complex of the desert locust, *Schistocerca gregaria*

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Abstract

The central complex is a group of modular neuropils in the insect brain with a key role in visual memory, spatial orientation, and motor control. In desert locusts the neurochemical organization of the central complex has been investigated in detail, including the distribution of dopamine-, serotonin-, and histamine-immunoreactive neurons. In the present study we identified neurons immunoreactive with antisera against octopamine, tyramine, and the enzymes required for their synthesis, tyrosine decarboxylase (TDC) and tyramine β -hydroxylase (TBH). Octopamine- and tyramine immunostaining in the central complex differed strikingly. In each brain hemisphere tyramine immunostaining was found in four neurons innervating the noduli, 12–15 tangential neurons of the protocerebral bridge, and about 17 neurons that supplied the anterior lip region and parts of the central body. In contrast, octopamine immunostaining was present in two bilateral pairs of ascending fibers innervating the upper division of the central body and a single pair of neurons with somata near the oesophageal foramen that gave rise to arborizations in the protocerebral bridge. Immunostaining for TDC, the enzyme converting tyrosine to tyramine, combined the patterns seen with the tyramine- and octopamine antisera. Immunostaining for TBH, the enzyme converting tyramine to octopamine, in contrast, was strikingly similar to octopamine immunolabeling. We conclude that tyramine and octopamine act as neurotransmitters/modulators in distinct sets of neurons of the locust central complex with TBH likely being the rate limiting enzyme for octopamine synthesis in a small subpopulation of TDC-containing neurons.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

ROLE OF AUTHORS

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: UH. MJA. Acquisition of data: JS, UB. Analysis and interpretation of data: UH. Drafting of the manuscript: UH. Critical revision of the manuscript for important intellectual content: UH, MJA. Obtained funding: UH, MJA. Administrative, technical, and material support: MJA, MM. Study supervision: UH.

Indexing terms

biogenic amines; immunocytochemistry; neuroanatomy; insect brain; neuromodulation; central complex

INTRODUCTION

Octopamine and tyramine are closely related trace amines with widespread occurrence in the nervous system of invertebrates (Evans, 1985; Agricola et al., 1988; Roeder, 1999, 2005; Pflüger and Stevenson, 2005; Farooqui, 2007; Busch et al., 2009). Both amines are synthesized from the amino acid tyrosine. Tyrosine decarboxylase (TDC) converts tyrosine into tyramine, which is further processed to octopamine by tyramine β -hydroxylase (TBH) (Monastirioti et al., 1996; Alkema et al., 2005; Cole et al., 2005). The role of octopamine as a central and peripheral neuromodulator of the insect nervous system has been studied intensely. Octopamine has a multitude of functions that largely resemble those of adrenaline and noradrenaline in vertebrates (Evans, 1985; Roeder, 1999; Verlinden et al., 2010). In the periphery, octopamine modulates neuromuscular transmission, the contraction of visceral muscles, virtually all sensory organs, and affects energy metabolism (Roeder, 2005; Farooqui, 2007; Verlinden et al., 2010). In the central nervous system, octopamine orchestrates rhythmic behaviors (Sombati and Hoyle, 1984; Claassen and Kammer, 1986; Vierk et al., 2009), mediates appetitive olfactory and visual conditioning (Giurfa, 2006; Unoki et al., 2006), enhances visual sensitivity (Kloppenburg and Erber, 1995; Bacon et al., 1995; Longden and Krapp, 2009), aggressiveness (Stevenson et al., 2000, 2005; Rillich and Stevenson, 2011; Certel et al., 2007; Hoyer et al., 2008; Potter and Luo, 2008; Zhou et al., 2008), and promotes wakefulness in *Drosophila* (Crocker et al., 2010). In contrast, tyramine has been regarded for a long time solely as the biosynthetic intermediate to octopamine synthesis, but a physiological role of its own is gaining experimental support. In the nematode *Caenorhabditis elegans*, tyramine acts as a genuine neurotransmitter demonstrated by immunostaining, analysis of synaptic mechanisms, and behavioral studies (Alkema et al., 2005; Pirri et al., 2009). In flies and locusts tyramine-immunoreactive neurons have been reported that lack octopamine immunostaining (Nagaya et al., 2002; Kononenko et al., 2009). Release of tyramine in locust brains (Downer et al., 1993) and from nerve terminals in locust oviducts and spermatheca has been demonstrated (Lange, 2009). G-protein coupled receptors with higher affinity to tyramine than to octopamine have been identified in various insect species including *Drosophila*, locust, moths, honeybee and cockroach, and were termed TyrRI by Verlinden et al. (2010). Members of a second class of tyramine receptors, termed TyrRII, that show virtually no cross-reactivity for octopamine have been characterized in *Drosophila* and *Bombyx* and are likely to be wide-spread (Cazzamali et al., 2005; Verlinden et al., 2010). Pharmacological studies in a variety of species and behavioral studies of TBH-mutants in *Drosophila* suggest distinct physiological roles for tyramine on flight motor activity (Fussnecker et al., 2006; Brembs et al., 2007; Vierk et al., 2009), locomotion (Saraswati et al., 2004), visceral muscle contractions (Lange, 2009), and sex pheromone production (Hirashima et al., 2007).

As a basis for functional studies on the roles of octopamine and tyramine in the central complex of the insect brain, we have used immunostaining techniques to study the cellular distribution of both amines in the central complex of the desert locust. We tentatively refer to these neurons as “octopaminergic” and “tyraminergic” but are aware of the fact that immunostaining does not provide conclusive evidence for a neuroactive role of these amines. The central complex is a group of midline spanning neuropils in the insect brain (Homberg, 2008). It consists of the protocerebral bridge, the upper and lower divisions of the central body, and a pair of ventral noduli (Fig. 1). The central complex is closely

connected to two adjacent brain areas, the lateral accessory lobes and the anterior lip (Fig. 1). Studies in locusts, grasshoppers, crickets, cockroaches, and fruit flies point to a role of the central complex in various aspects of motor control, spatial orientation, and visual memory (Strauss, 2002; Liu et al., 2006; Heinze and Homberg, 2007; Triphan et al., 2010). In desert locusts, single-cell recordings demonstrated prominent responses to polarized light and suggest that the central complex serves a role as an internal celestial compass (reviewed by Homberg et al., 2011). The neuroarchitecture of the locust central complex has been studied in detail (Williams, 1975; Müller et al., 1997; Heinze and Homberg, 2008). It is composed of systems of columnar neurons, pontine neurons, and tangential neurons which together give rise to a regular arrangement of vertical columns and horizontal layers in the subdivisions of the central complex. Regular chiasmal fiber crossings between the right and left hemispheres are a unique feature of this brain area. In all insect species studied, the central complex shows prominent octopamine immunostaining (locust: Konings et al., 1988; Kononenko et al., 2009; honeybee: Kreissl et al., 1994; Sinakevitch et al., 2005; flies: Monastirioti et al., 1995; Sinakevitch and Strausfeld, 2006; Busch et al., 2009; cockroach: Sinakevitch et al., 2005). The distribution of tyramine- and octopamine immunoreactivities differ in the locust central complex (Kononenko et al., 2009). The antisera against both amines label potential release sites of octopamine and tyramine intensely, while cell bodies are more weakly stained which has hampered the identification of tyraminergeric and octopaminergic neurons in the locust nervous system. In addition, as a precursor tyramine is present in octopamine releasing cells and antisera against both closely related amines usually show some crossreactivity. This further impairs a conclusive distinction between octopaminergic and tyraminergeric cell populations in the locust CNS. In *C. elegans*, tyramine β -hydroxylase (TBH) is expressed in a subset of cells that express tyrosine decarboxylase (TDC) facilitating the identification of octopaminergic and tyraminergeric cells (Alkema et al., 2005). To determine whether tyramine functions independently of octopamine in the locust brain we performed a comparative immunohistochemical analysis with antisera against tyramine, octopamine and their biosynthetic enzymes, TDC and TBH. Our analysis reveals novel and distinct tyraminergeric and octopaminergic neurons in the central complex of the locust.

MATERIAL AND METHODS

Animal preparation

Desert locusts (*Schistocerca gregaria*) were raised in crowded colonies at the Universities of Konstanz and Marburg (28°C, 12L:12D photoperiod). Experiments were performed on sexually mature adult males and females 2–3 weeks after adult ecdysis. Animals were isolated and kept in individual cages 1–2 days prior to dissection.

Antibody characterization

Two antisera against octopamine, an antiserum against tyramine, and antisera against tyrosine decarboxylase (TDC) and tyramine β -hydroxylase (TBH) were used for immunostaining (Table 1). A polyclonal antiserum raised in rabbit against glutaraldehyde conjugates of octopamine and thyroglobuline was obtained from Dr. H.G.B. Vullings (University of Utrecht, The Netherlands). The specificity of the antiserum has been characterized by Spörhase-Eichmann et al. (1992). Cross-reactivity of the antiserum in dot blot immunoassays was < 5% with tyramine, < 0.33% with dopamine and < 0.17% with noradrenaline and serotonin. A second polyclonal antiserum raised in rabbit against octopamine-thyroglobuline conjugates was obtained from Dr. M. Eckert (University of Jena, Germany). Dot blot immunoassays again showed some crossreactivity with tyramine (< 1%) but none with dopamine, serotonin, and norepinephrine (Eckert et al., 1992). For anti-tyramine immunostaining, a polyclonal antiserum, raised in rabbit (# AB124) against

glutaraldehyde conjugates of p-tyramine and N- α -acetyl-L-lysine-N-methylamide, was purchased from Millipore (Billerica, MA, USA). According to the manufacturer, crossreactivities determined using ELISA or RIA was 0.125% with conjugates of octopamine and bovine serum albumin (BSA), <0.0025% with dopamine-BSA, and <0.002% with tyrosine-BSA. In immunostaining of *S. gregaria* brain sections, we tested the specificities of the octopamine and tyramine antisera by liquid-phase preadsorption of the diluted antisera with various concentrations (100 nM - 100 μ M) of tyramine and octopamine conjugated through glutaraldehyde with bovine serum albumin (BSA). Preadsorption of the diluted octopamine antisera with 1 μ M octopamine-BSA or 100 μ M tyramine-BSA abolished all immunostaining on locust brain sections. The relative concentrations of antigen that resulted in complete block of immunostaining, therefore, suggests about 1% crossreactivity of the octopamine antisera with tyramine. Likewise, preadsorption of the anti-tyramine serum with 10 μ M tyramine-BSA abolished all immunostaining. In contrast, preincubation of the anti-tyramine antiserum with up to 100 μ M octopamine-BSA led to reduction in staining intensity but did not completely block immunostaining.

As independent indicators for the presence of tyramine and octopamine, we used antisera against the enzymes involved in tyramine and octopamine biosynthesis, TDC and TBH. The TDC antiserum was raised in rabbit against fusion proteins of glutathione-S-transferase (GST) and amino acids 81–361 from TDC-1 of the nematode *C. elegans*. This part of the protein is highly conserved between *D. melanogaster* and *C. elegans* TDC (Alkema et al., 2005). The antibody was purified against gel purified recombinant protein and absorbed against recombinant GST on Western blot strips. Immunostaining of *C. elegans* nervous tissue revealed the same staining pattern as that obtained with an antiserum against GST-TDC-1A (amino acids 534–650) (Alkema et al., 2005). Staining was absent in *tdc-1* deletion mutants (Alkema, unpublished). Western blot analyses from total protein of *C. elegans* revealed a double band around 75 kDa molecular weight corresponding to the predicted size of the two splice variants, TDC-1A (73.2 kDa) and TDC-1B (79.7 kDa). The doublet is absent in Western blot analyses of protein extracts from *tdc-1* mutants. In Western blots from homogenized locust brains the TDC-1 antibody revealed a major band of approximately 69 kDa.

The antiserum against TBH was raised in rat against amino acids 103–553 from *Drosophila melanogaster* TBH (Monastirioti et al., 1996). For immunization, the protein was purified after heterologous expression in *E. coli*. In *D. melanogaster*, the distribution of anti-TBH closely resembled the pattern of octopamine immunostaining, and *T β h* null mutations resulted in complete loss of octopamine- and a strong increase in tyramine levels in the brain (Monastirioti et al., 1996). Western blot analysis of wild type flies revealed a single band corresponding to the predicted 76 kDa TBH protein (Monastirioti et al., 1996). In Western blots from locust brains, following standard procedures we found a major band at 65 kDa.

Immunounostaining

Octopamine- and tyramine immunostaining—Immunostaining was performed on free-floating sections by use of the indirect peroxidase technique or the peroxidase anti-peroxidase (PAP) technique (Sternberger, 1979). Animals were isolated from the crowded cages for two days prior to tissue preparation. After anesthetizing the animals by cooling, their brains were dissected in cold (4°C) freshly prepared fixative consisting of 2.5% glutaraldehyde and 1% (for anti-tyramine 0.5%) sodium metabisulfite in phosphate buffer (0.1 M, pH 7.4) or in a mixture containing 6.25% glutaraldehyde, 75% saturated picric acid, 5% glacial acetic acid and 1% sodium metabisulfite. After fixation for 2–3 hours, brains were embedded in gelatin/albumin and sectioned in 0.1 M phosphate buffer containing 1% Triton X-100 (Sigma, Deisenhofen, Germany) and 1% (anti-octopamine) resp. 0.5% (anti-

tyramine) sodium metabisulfite at 30–40 μm with a Vibratome (Technical Products, St. Louis, Mo., USA) or a vibrating blade microtome (Leica, VT 1000S; Leica, Nussloch, Germany). After rinsing in sectioning buffer, double bonds were saturated by incubating the sections for 10–15 minutes in 0.13 M sodium borohydride in phosphate buffer containing 1% Triton X-100. After thorough rinsing in PBS (0.45 M NaCl/1% Triton X-100 in 0.1 M phosphate buffer, pH 7.4), the sections were preincubated for 1 hour in 10% normal goat serum in PBS and subsequently in primary antiserum overnight at room temperature. Both octopamine antisera raised in rabbit were diluted at 1:5,000 and the anti-tyramine antiserum at 1:4,000 in PBS containing 1% Triton X-100. Secondary antiserum (goat-anti rabbit IgG; Sigma) was applied for 1 hour at a dilution of 1:40 and rabbit PAP (Dako; Hamburg, Germany) for 1 hour at 1:300. The sections were subsequently treated with a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.3 mg/ml) in 0.1 M phosphate buffer, pH 7.4, and H_2O_2 (0.045%). The sections were finally mounted on chrome alum/gelatin-coated glass slides, dehydrated, cleared in xylenes, and embedded in Entellan (Merck, Darmstadt, Germany) under glass coverslips.

TDC-1- and TBH immunostaining—For anti TDC-1- immunostaining, brains were fixed overnight in freshly prepared Zamboni's fixative (4% paraformaldehyde, 7.5% picric acid in 0.1 M phosphate buffer, pH 7.4). For anti TBH immunolabeling, freshly prepared Bouin's fixative (3 ml saturated picric acid, 1 ml formalin, 0.2 ml glacial acetic acid) was used. After fixation, brains were embedded in gelatin/albumin and sectioned at 30 μm with the vibrating blade microtome in 30 μm sections. Immunostaining was performed on the free-floating sections by the indirect immunoperoxidase technique. The TDC antiserum was diluted at 1:50 and the TBH antiserum at 1:300. Primary antisera were diluted in 0.1 M Tris HCL/0.3 M NaCl, pH 7.4, containing 0.5% Triton X-100 (Sigma) and 2% normal goat serum. They were applied to the sections for at least 18 hours at room temperature. Horseradish peroxidase (HRP) conjugated goat-anti-rabbit (Sigma, Deisenhofen, Germany) was used as secondary antiserum for TDC immunostaining and HRP-conjugated goat anti-rat as secondary antiserum for TBH immunostaining. Secondary antisera were applied for 1 hour at a dilution of 1:300. After rinsing, the sections were subsequently treated for 5–20 minutes with a solution of 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.3 mg/ml) in 0.05 M Tris-HCl, pH 7.4 with 0.3% nickel ammonium sulfate and 0.045% H_2O_2 . The sections were finally mounted and embedded under glass cover slips as described above.

Neuron reconstruction and image processing

Neuropil structures and immunostained neuron ensembles were reconstructed from serial, immunocytochemically stained sections with a camera lucida attachment on a Leitz compound microscope equipped with a 40 $\times$ objective. Selected images from the preparations were captured using a Zeiss Axioskop compound microscope equipped with a digital camera (ProgRes C12^{plus}, Jenoptik, Jena, Germany). Images were processed to adjust brightness and contrast using Adobe Photoshop CS2 software (Adobe Systems; San Jose, CA). Figures 4E, 6F, and 7F are superimposed images from 2 (Figs. 4E, 7F), resp. 4 (Fig. 6F) adjacent microtome sections. The complete images were obtained in Adobe Photoshop by montaging adjacent areas from the sections using the “darken” function of Photoshop. Positional information is given with respect to the body axis of the animal.

RESULTS

For morphological identification of octopamine- and tyramine-immunostained neurons innervating the central complex of the locust brain, we have partly reconstructed the labeled neurons from serial immunostained brain sections. Based on cell body position and fiber projections we distinguished 6 groups of tyramine-immunostained neurons, termed T1–T6

and two types of octopamine-immunostained neurons termed O1 and O2. With both antisera, presynaptic terminals were most intensely stained, while cell bodies were more weakly labeled. Although the patterns of immunostaining for octopamine and tyramine in the central complex and associated brain areas showed very little overlap, cross-reactivity between both antisera cannot be completely excluded. Therefore, we additionally performed immunostaining with antisera against TDC, the enzyme converting tyrosine to tyramine, and against TBH, the enzyme mediating biosynthesis of octopamine from tyramine. Double-label experiments combining the TBH or TDC antiserum with one of the three other antisera in the same preparation were unsuccessful owing to mutually incompatible fixation protocols. Therefore, we had to rely on detailed comparison of neuronal morphologies to assess whether the same neurons were labeled with the different antisera.

Tyramine immunostaining

Several subunits of the locust central complex and its associated brain structures showed dense tyramine immunostaining (Fig. 2). The anterior lip in front of the central body was densely supplied by immunostained processes and terminals (Fig. 2C). Most of them originated from neuronal somata in the anterior inferior medial and lateral protocerebrum (Fig. 2F). In total, three cell clusters, termed T1–T3, in the soma ring surrounding the medial lobes of the mushroom bodies could be distinguished (Figs. 2F, 3). An anterior lateral cluster (T1) consisted of 7–9 bilateral pairs of somata, a more medial cluster (T2), of 5 somata and a small medio-ventral cluster (T3), of three somata. Owing to the large number of stained neurons in the inferior protocerebrum, their projections could not be traced individually. Neurites from the three clusters passed anteriorly and posteriorly around the medial lobes of the mushroom body. Side branches extended to various areas in the superior median and lateral protocerebrum (Fig. 3A). The neurons formed a dense fiber meshwork in the anterior lip, where many fibers crossed the brain midline (Fig. 2C). A few processes entered the upper division of the central body and formed fine, beaded processes confined to ventral parts of layer Ib of the upper division of the central body (Fig. 2B, arrowheads). Some neurites from T1–T3 neurons extended posteriorly around the medial lobes and entered the dorsal and ventral shells of the lateral accessory lobes. Several of these projections were smooth and faintly stained while others were strongly labeled and varicose (Fig. 2B). Other axonal projections continued from the anterior lip via the medial accessory lobe to posterior lateral brain areas (Fig. 3A, arrows). Except for staining in layer Ia, the lower and upper divisions of the central body were devoid of tyramine immunostaining (Fig. 2D).

The noduli were densely supplied by immunostained terminals, consistent with data from Kononenko et al. (2009). Innervation of the noduli originated from 4 neurons per hemisphere, termed T4. They had cell bodies medial and posterior to the antennal lobes (Fig. 2I), close to a group of large octopamine/tyramine immunoreactive somata near the oesophageal foramen (Fig. 2I), termed OA1/TA by Kononenko et al. (2009). Primary neurites from the T4 neurons entered the lateral accessory lobe and gave rise to faintly stained, smooth and, therefore, probably dendritic side branches that extended to the dorsal and parts of the ventral shells of the LAL (Fig. 3B). Axonal fibers continued through the isthmus tract to the ipsilateral nodulus, but did not cross the midline of the brain. Fine beaded terminals were concentrated in the lower units of the noduli and more coarse and varicose terminals in layers I and II of the upper units of the noduli (Figs. 2E, 3B). Differential supply of the different nodular units by the four fibers is likely but could not be confirmed with certainty.

The protocerebral bridge was densely supplied by immunostained neurons which could be traced in an ensemble reconstruction (Figs. 2H, G, 3C). Their cell bodies resided in two cell clusters, one dorso-laterally and posteriorly from the bridge consisting of 4–7 neurons per

hemisphere (T5 neurons, arrowheads in Fig. 3C) and a cell cluster of 8 neurons posteriorly from the lateral arms of the bridge (T6 neurons, double arrowheads in Fig. 3C). Primary neurites from the two cell groups gave rise to extensive smooth and weakly stained processes in large areas of the posterior median brain, but did not invade the posterior optic tubercles (Fig. 2G). Axonal fibers entered the posterior-lateral tips of the protocerebral bridge adjacent to the posterior-optic tubercle-protocerebral bridge tract. Immunostained fibers traversed the protocerebral bridge tangentially and gave rise to numerous beaded processes throughout the bridge.

Octopamine immunostaining

Both antisera against octopamine provided virtually identical results. Immunostaining was much more sparsely distributed in the central complex and differed strikingly from tyramine immunostaining (Fig. 4). Immunoreactivity in the anterior lip, which was densely supplied by tyramine-immunoreactive neurons, was nearly absent with only a few terminals and two small fibrous processes visible (Fig. 4B). In correspondence with this T1-3 cell bodies in the inferior median cell body ring were not octopamine-immunostained. The lower division of the central body was sparsely invaded from ventral directions by fine beaded processes (Fig. 4A) that originated from a bilateral pair of fine fibers in the lateral accessory lobes, but their further origin could not be determined. A more prominent meshwork of beaded processes invaded the upper division of the central body. Fibrous staining was especially concentrated in layers Ia and IIa but also invaded the noduli (Fig. 4C, D). This fiber meshwork largely originated from two bilateral pairs of neurites that could be identified as side branches of a pair of ascending fibers from the circumoesophageal connective (Fig. 5A). Although the cell bodies of these neurons were not identified, they were tentatively termed O1 neurons. Their fibers entered the brain at the inner posterior side of the tritocerebrum. They ascended near the midline of the brain and, ventrally from the central body, turned dorso-laterally and anteriorly toward the calyces of the mushroom bodies. Side branches entered the central body laterally and projected along the dorsal face of the upper division of the central body (Figs. 4D, 5A). Numerous beaded processes extended from these fibers into the upper division. Some of these continued into the noduli. The noduli were invaded sparsely by immunostained processes, quite unlike the dense concentration of terminals seen in tyramine immunostaining. Stained processes were concentrated in the lower units and in layers I and II of the upper units of the noduli (Fig. 4C). Some of these originated from the processes in the upper division of the central body (Fig. 5A), but additional processes seemed to enter the noduli from lateral directions. The lateral accessory lobes showed fine granular immunostaining of unknown origin, which was particularly dense in the lateral triangle and median olive (Fig. 4A).

The protocerebral bridge showed uniform fine beaded immunostaining. Neither tracts extending from the tips of the bridge nor tangentially running fibers showed immunostaining. Instead, staining was traced to a bilateral pair of neurites that invaded the protocerebral bridge along its ventral surface (Figs. 4E, 5B). Cell bodies of these neurons, termed O2, were among the OA1/TA cell cluster near the oesophageal foramen (Kononenko et al., 2009). A bilateral pair of neurites from the O2 neurons ascended on each side of the midline (Fig. 5B). Each fiber bifurcated and sent a collateral across the brain midline. The two collaterals ramified further and invaded both hemispheres of the bridge. Before bifurcation, a major, possibly dendritic side branch extended toward lateral brain areas but could only be traced for a short distance (Fig. 5B).

TDC immunostaining

Because tyramine- and octopamine immunostaining differed substantially in their distribution in the central complex we sought to validate these results through independent

markers. As such we employed an antiserum against tyramine β -hydroxylase (TBH) from the fruit fly *Drosophila* and an antiserum against tyrosine decarboxylase (TDC) from the nematode *C. elegans*.

The anti-TDC antiserum gave rise to uniform dense immunostaining in all neuropil structures of the locust brain, suggesting that it recognizes epitopes associated with synaptic areas (Fig. 6). In addition, more intense fibrous immunostaining was present in a small number of interneurons in the brain. In the central complex, this fibrous staining closely resembled the combined pattern of tyramine- and octopamine immunostaining. As seen in tyramine immunostaining, a dense plexus of fibrous staining was present in the anterior lip (Fig. 6B). Like in tyramine immunostaining, it originated from three clusters of cell bodies in the anterior inferior median and lateral protocerebrum corresponding in position and number to the T1–T3 clusters of tyramine immunostained neurons (not shown). Stained fibers from this plexus could be traced to various areas in the central brain, including the lateral and medial accessory lobes (Fig. 6A) and the superior protocerebrum. The noduli were supplied by four bilateral pairs of neurons most likely identical to the tyramine positive T4 neurons. Their cell bodies were close to two clusters of large somata near the oesophageal foramen (Fig. 6C), and their main fibers passed through the lateral accessory lobe to the noduli. The staining pattern in the noduli showed striking similarity to that of tyramine-immunostained T4 neurons, including dense granular staining in the lower units of the noduli, coarse varicose staining in layers I and II of the upper units and lack of stained terminals in layer III (Fig. 6D). The protocerebral bridge was densely supplied by processes from horizontally projecting fibers that crossed the midline of the bridge (Fig. 6F). Similar to T5/T6 neurons, fibers from neurons with cell bodies in two clusters posteriorly and dorso-laterally from the bridge entered the lateral tips of the bridge. Fine, presumably dendritic ramifications of these neurons extended widely in the posterior protocerebrum but did not invade the posterior optic tubercles. In addition, and similar to O2 neurons, bilateral pairs of fibers entered the two arms of the bridge from their ventral side (Fig. 6F). These neurites could not be traced to their cell bodies, but as seen in tyramine- and octopamine immunostaining, groups of large somata near the oesophageal foramen, presumably the OA1/TA cells, were intensely stained (Fig. 6C). Finally, similar to O1 octopamine neurons, pairs of processes from fibers ascending from the ventral nerve cord near the brain midline passed along the upper face of the upper division of the central body and sent processes into the upper division (Figs. 6A, E). Owing to high background staining, their terminal targets in the central body could not be determined.

TBH immunostaining

In contrast to TDC immunostaining, TBH immunolabeling had a distinct beaded appearance throughout the brain, with only weak staining in fibrous processes and cell bodies, suggesting that the enzyme is largely concentrated in presynaptic terminals (Fig. 7). This is consistent with the observation that TBH is localized to synaptic vesicles in *C. elegans* and *Drosophila* (Alkema et al., 2005; Koon et al., 2011). A sparse meshwork of processes and terminals, closely resembling the pattern of octopamine labeling, showed TBH immunoreactivity (Figs. 7, 8). The anterior lip was largely free of immunostaining (Fig. 7B) and no cell bodies were immunostained in the inferior medial and lateral protocerebrum. Strongly immunoreactive beaded processes were especially prominent in the upper division of the central body. As in O1 neurons, these processes originated from two bilateral pairs of ascending fibers from the ventral nerve cord that gave rise to immunostained fibers along the lateral accessory lobes and peduncle of the mushroom body. They sent two side branches along the dorsal face of the central body that gave rise to beaded staining in the central body (Fig. 7A, E). The lateral accessory lobes were sparsely stained, but like in octopamine immunostaining, the lateral triangles and median olives were more densely labeled (Fig.

7A). The noduli were sparsely invaded by immunostained processes, like in octopamine immunostaining with terminals concentrated in the lower units and layers I and II of the upper units (Fig. 7D). The protocerebral bridge was, likewise, only sparsely stained. Strikingly similar to the octopamine positive O2 neurons, the TBH-immunoreactive innervation of the bridge originated from fibers that entered the two hemispheres of the bridge from its ventral side (Figs. 7F, 8). They could, however, not be traced to their cell-bodies but groups of large somata, probably identical to OA1/TA neurons, were again labeled adjacent to the oesophageal foramen (Fig. 7C).

DISCUSSION

We have identified through ensemble reconstructions the morphologies of tyramine- and octopamine-immunoreactive neurons innervating substructures of the central complex in the brain of the desert locust. The stained cell types differ from neurons labeled with serotonin- (Homberg, 1991), dopamine- (Wendt and Homberg, 1992), and histamine antisera (Gebhardt and Homberg, 2004), demonstrating that immunostaining reported here is not caused by crossreactivity with other amines. Although tyramine is the precursor molecule of octopamine, distinct sets of neurons of the central complex were immunostained by antisera against the two amines (Fig. 9). Immunostaining for TDC, the enzyme that converts tyrosine into tyramine, revealed a combination of neurons stained with the octopamine- and tyramine antisera, whereas an antiserum against TBH, the enzyme that catalyzes synthesis of octopamine from tyramine, resulted in staining strikingly similar to octopamine labeling (Fig. 9). Because tyramine is the precursor of octopamine, this implies nearly quantitative conversion of tyramine to octopamine in the O1 and O2 octopaminergic neurons. The data suggest that tyramine and octopamine act independently as neurotransmitters/modulators in different types of neuron of the locust central complex. This situation is apparently not true for all labeled neurons in the locust brain. As shown here and partly by Kononenko et al. (2009), the cluster of OA1/TA neurons near the oesophageal foramen is stained with all antisera used here, and accordingly, their projections in the optic lobe both show tyramine- and octopamine labeling.

Octopamine

Octopamine serves a modulatory role in the insect nervous system largely analogous to that of norepinephrine and epinephrine in vertebrates. In various insect species octopamine enhances aggressiveness, promotes wakefulness, orchestrates rhythmic behaviors, increases sensitivity to sensory stimuli, and mediates the reinforcement signal in appetitive conditioning (Pflüger and Stevenson, 2005; Farooqui, 2007; Verlinden et al., 2010). In the central complex, a brain area involved in motor control and visual signal processing, octopamine immunostaining has been demonstrated in honeybees (Kreissl et al., 1994; Sinakevitch et al., 2005), flies (Monastirioti et al., 1995; Sinakevitch and Strausfeld, 2006; Busch et al., 2009), locusts (Konings et al., 1988; Kononenko et al., 2009), and the American cockroach (Sinakevitch et al., 2005). In *Drosophila* and the migratory locust *Locusta migratoria*, ascending midline neurons from the suboesophageal ganglion innervate wide brain areas including the upper division of the central body (Bräunig, 1991; Busch et al., 2009). In *Drosophila* a bilateral pair of ventral median neurons of the suboesophageal ganglion, termed OA-VPM3, which were identified using the Flp-out technique to GAL4 lines of putatively octopaminergic neurons, innervates the fan-shaped body (equivalent to the upper division of the central body). The OA-VPM3 neurons have spiny ramifications ventrally around the oesophageal foramen and send a single ascending fiber into the brain which gives rise to varicose processes in the upper division of the central body (termed fan-shaped body in flies), the superior median protocerebrum, and the calyces of the mushroom body. Diffuse innervation of the fan-shaped body by octopamine-immunostained midline

neurons of the suboesophageal ganglion was also suggested for the blowfly (Sinakewitch and Strausfeld, 2006). Through cobalt injection, Bräunig (1991) identified a midline neuron in the suboesophageal ganglion of the migratory locust (*Locusta migratoria*) with ascending projections to the central body strikingly similar to the ascending projections of the O1 neurons innervating the central body shown here. The neuron in the migratory locust, termed DUM SA1, sent ascending fibers in both connectives to the brain and innervated the lateral accessory lobes, the upper division of the central body, parts of the noduli, the calyces and various areas in the superior protocerebrum. Several details in the innervations pattern, including the common course of the ascending processes, dense innervations especially of the lower units of the noduli and invasion of the upper division of the central body from its dorsal surface strongly suggest that the O1 neurons stained here are the *Schistocerca* homologues of the *Locusta* DUM SA1 neurons. The presence of pairs of octopaminergic neurites in each connective found in *Schistocerca*, however, suggests that DUM SA1 might not be an unpaired neuron but rather a pair of octopamine-containing neurons with ascending projections as in *Drosophila*.

Clusters of large octopamine-immunoreactive neurons near the oesophageal foramen have been described in all species studied. We show that one neuron (O2) from that cluster (group C1 in Stevenson and Spörhase-Eichmann, 1995; OA1/TA in Kononenko et al., 2009) gives rise to immunostaining in the protocerebral bridge. Previous studies in locusts (Stern et al., 1995; Bacon et al., 1995; Stevenson and Spörhase-Eichmann, 1995; Stern, 1999; Kononenko et al., 2009) showed that many of these neurons send axonal fibers into the medulla and lobula of the optic lobe. In the migratory locust, a neuron of that group, termed PM5, was labeled through intracellular cobalt injection and innervates the protocerebral bridge in a manner strikingly similar to that of the O2 neuron shown here (Stern, 1999). PM5 has dendritic ramifications in the antennal mechanosensory and motor center and posterior slope and, in addition to bilateral innervation of the protocerebral bridge, sends fibers into the ocellar tracts and wide projections into the ipsilateral medulla and contralateral medulla and lobula. The *Schistocerca* homologue of PM5 is, therefore, a strong candidate giving rise to octopamine labeling in the bridge, although collaterals into the ocellar nerves and toward the contralateral optic lobe have not been revealed in our preparations. Innervation of the protocerebral bridge by octopamine-containing neurons near the oesophageal foramen has also been found in the sphinx moth *Manduca sexta* and in the fly *Drosophila* (Homberg, unpublished; Busch et al., 2009). The *Drosophila* neuron, termed OA-AL2i1, has again been identified through the Flp-out technique. It has its cell body in the soma rind medially from the deutocerebrum, innervates neuropil in the posterior slope close to the oesophageal foramen, sends a process into the protocerebral bridge and an axonal fiber into the ipsilateral lobula complex and medulla of the optic lobe.

In the honeybee, flies and the American cockroach, additional octopamine-immunostained neurons of the central complex were described, which were not found in *Drosophila* with the Flp-out technique (Busch et al., 2009). These include tangential neurons of the protocerebral bridge in bees and cockroaches (Sinakevitch et al., 2005), columnar neurons connecting the protocerebral bridge to the lower division of the central body in bees and flies (Sinakevitch et al., 2005; Sinakevitch and Strausfeld, 2006), and processes connecting the lower division of the central body to the inferior lateral protocerebrum in flies. None of these neuronal systems showed octopamine labeling in locusts.

Tyramine

The distributions of octopamine and tyramine differ strikingly in the locust central complex. Our observations are consistent with the findings by Kononenko et al. (2009). However we identified additional cell clusters that contribute to tyramine immunostaining in the central complex. T5 and T6 neurons, collectively termed TA2 by Kononenko et al. (2009) give rise

to immunostaining in the protocerebral bridge. Although the morphologies of individual neurons of these types still has to be elucidated, they can be regarded as a novel type of tangential neuron (TB neuron) of the protocerebral bridge, distinct from the hitherto characterized TB1 and TB2 neurons that connect the protocerebral bridge to the posterior optic tubercles (Heinze and Homberg, 2007, 2009). The noduli are supplied by four pairs of T4 neurons with cell bodies near the oesophageal foramen and not, as proposed by Kononenko et al. (2009) by the OA3/TA cell cluster dorso-lateral to the antennal lobe. T4 neurons share cell body position and major fiber trajectory with many tangential neurons of the central body, including TL2-4 tangential neurons of the lower division of the central body (Müller et al., 1997), but instead of invading particular layers of the central body they specifically target sublayers of the ipsilateral nodulus. Finally, the tyramine-immunoreactive fiber plexus in the anterior lip with partial innervation of the upper division of the central complex was interpreted by Kononenko et al. (2009) as being in the lower division of the central body. We identified three tyramine-immunoreactive clusters of cell bodies (T1–T3), comprising 15–17 cell bodies, in contrast to the previously reported two cell bodies (cluster TA9) in a position similar to the T2 or T3 cell cluster. The lack of tyramine immunostaining in octopamine-immunoreactive arborizations in the central complex suggests that TBH might be the rate limiting enzyme in those putatively octopaminergic neurons. Whereas TDC is localized to cytoplasm, TBH is concentrated in synaptic vesicles (Alkema et al., 2005; Koon et al., 2011). The spatial distribution of these enzymes may drive the complete conversion of tyramine to octopamine in synaptic vesicles, resulting in concentration of tyramine below the detection threshold with immunostaining techniques used here. This may, however, not be true for the cell bodies of the labeled octopamine-immunoreactive cells: the OA1/TA cell cluster near the oesophageal foramen and midline clusters in the suboesophageal ganglion showed both octopamine- and tyramine immunoreactivity (Kononenko et al., 2009).

TDC and TBH

The biosynthesis of octopamine requires two steps, decarboxylation of tyrosine by tyrosine decarboxylase (TDC) leading to tyramine, and hydroxylation of tyramine by tyramine β -hydroxylase (TBH) to form octopamine. Despite the importance of these enzymes for controlling levels of tyramine and octopamine, very little is known about the regulations and modifications of their activities. The distribution of both enzymes is reported here for the first time in an insect brain. The anti-TDC antiserum was generated against a part of TDC-1 from *C. elegans* that shows more than 60% identity with neurally expressed *Drosophila* TDC2 and the predicted orthologous proteins from the honeybee and the mosquito *Anopheles gambiae* (Alkema et al., 2005; Cole et al., 2005). On locust brain sections, the antiserum apparently cross reacts with an unknown synaptic epitope, thus labeling all neuropil areas of the locust brain, but in addition showed stronger immunostaining of neurons that were identified as tyramine- and octopamine-immunoreactive, and therefore presumably contained locust TDC.

The anti-TBH antiserum was generated against *Drosophila* TBH (Monastirioti et al., 1996). Immunostaining in the locust central complex matched octopamine immunoreactivity, and suggests that a small fraction of tyramine-synthesizing TDC neurons contains TBH for biosynthesis of octopamine. Judged from deduced amino acid sequences of *Drosophila* and honeybee TBH and biochemical properties of TBH studied in sphinx moths and bees, TBH is closely related to mammalian dopamine β -hydroxylase (DBH; Lehmann et al., 2000; 2006), which mediates hydroxylation of dopamine to produce noradrenaline. The two enzymes dopa decarboxylase (DDC) and DBH can, therefore, be regarded as the vertebrate counterparts of TDC and TBH, with DDC being expressed in dopaminergic neurons, and DDC and DBH, in noradrenergic cells.

Functional implications

Comparison of immunostaining for tyramine, octopamine, TDC, and TBH suggests that distinct systems of neurons of the locust central complex are tyraminergergic and octopaminergic (Fig. 9). For the first time in an insect nervous system we directly compared immunostaining of the biosynthetic enzymes TDC and TBH that are required for the synthesis of tyramine and octopamine. Like in the nematode *C. elegans* our findings suggest that TBH is expressed in a subset of TDC expressing cells. This supports earlier evidence in locusts and other insects that tyramine, in addition to being the precursor of octopamine, serves a neurotransmitter role of its own in the insect nervous system (Downer et al., 1993; Nagaya et al., 2002; Kononenko et al., 2009; Lange, 2009; Verlinden et al., 2010). Ramifications of the stained neurons in the central complex are predominantly of beaded or varicose appearance suggesting that both amines are released and may modulate neural activity in the central complex. In desert locusts, and probably other insects as well, the central complex houses an internal sky compass that is likely used for spatial orientation during flight and walking (Sakura et al., 2007; Homberg et al., 2011; Merlin et al., 2012). It has been hypothesized that specific navigational tasks and motivational states may be important for gain control in sensory signal processing in the central complex (Sakura et al., 2007; Heinze et al., 2009; Merlin et al., 2012). The ascending projections of octopamine containing O1 and O2 neurons to the protocerebral bridge and central body may be one way to enhance orientation-related signal processing during flight, walking or other behaviors. The well characterized physiological properties of many central-complex neurons, likewise, provide the prospect of studying the neuronal effects of tyramine on signal processing in this brain structure.

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Figure 1.

(A) Frontal diagram of the brain and (B) schematic sagittal section through the central complex of the desert locust. The upper division of the central body (CBU) is divided into three layers termed I, II and III. Layers I and II are divided further into layers Ia, Ib and IIa, IIb. Likewise, three layers, termed I, II, III can be distinguished in the upper unit of the nodulus (NU). AL, antennal lobe; AnL, anterior lip; Ca, calyx of the mushroom body; CBL, lower division of the central body; LAL, lateral accessory lobe; mL, vL, medial and vertical lobe of the mushroom body; NL, lower unit of the nodulus; P, pedunculus; p, posterior; PB, protocerebral bridge; TC, tritocerebrum. Scale bars: 200 μ m in A, 50 μ m in B.

Figure 2.

Tyramine immunostaining in the central complex of the desert locust. (A) Schematic sagittal diagram through the central complex illustrating the planes of sections in B, C, D, and E. (B) Frontal section showing tyramine immunoreactivity originating from T1, T2, and T3 neurons in the dorsal and ventral shells of the lateral accessory lobe (LAL-DS, LAL-VS) and anterior aspects of layer Ia of the upper division of the central body (CBU, arrowheads). The anterior chiasma of the central complex (aCh) is devoid of immunostaining. Large immunostained fibers pass around the central complex via the medial accessory lobe (arrows). P, pedunculus of the mushroom body. (C) Immunostaining in T1-3 neurons in the anterior lip (AnL) and layer I of the CBU. The anterior bundles (AB) are largely free of immunostaining. (D) Layers IIB and III of the CBU and the lower division of the central body (CBL) are free of immunostaining. Arrowheads point to four bilateral pairs of immunostained fibers from T4 neurons that supply the noduli. (E) Tyramine immunostaining in the noduli. The lower units (NL) show dense uniform staining, while layers I and II of the upper units (I, II) are invaded by varicose processes. Layer III is largely

free of immunostained terminals. (F) Frontal section near the anterior surface of the brain, showing tyramine immunostaining in three clusters of neurons (T1, T2, T3). They give rise to the dense fiber plexus in the anterior lip shown in C. vL, vertical lobe of the mushroom body. (G) Frontal section through the posterior protocerebrum showing widely arborizing smooth processes from T5 and T6 neurons that innervate the protocerebral bridge (arrowheads). (H) Immunostaining in the protocerebral bridge (PB), frontal section. Large fibers project ventrally along the longitudinal axis of the bridge and give rise to beaded terminal specializations. (I) Bilateral clusters of large tyramine-immunoreactive somata near the oesophageal foramen (Oe). Small somata associated with these clusters (arrowheads) are those of T4 neurons, Scale bars = 100 μm in B,C,D,G,H,I; 50 μm in E; 200 μm in F.

Figure 3.

Frontal reconstructions of tyramine-immunoreactive neurons associated with the central complex. (A) Partial reconstruction of fiber trajectories that contribute to immunostaining in the anterior lip in front of the central body. Neurites originate from three clusters of cell bodies in the anterior soma ring of the brain, termed T1, T2 and T3. Neurites pass around the medial lobes of the mushroom bodies (mL) to the anterior lip and continue to wide areas in the superior lateral protocerebrum (SLP) and superior median protocerebrum (SMP). Collaterals project to deep brain areas via the median accessory lobe (mAL), or invade the lateral accessory lobes (arrowheads). AL, antennal lobe; Oe, oesophageal foramen; vL, vertical lobe. (B) Four bilateral pairs of T4 neurons with cell bodies near the oesophageal foramen (Oe). Their neurites run fasciculated through the lateral accessory lobe (LAL) and 29 give rise to terminal ramifications in sublayers of the ipsilateral nodulus (No). Fine, weakly stained processes extend from their main fibers into lateral areas of the lateral accessory lobe. (C) Two groups of neurons (T5, T6) with wide ramifications in the posterior

protocerebrum send axonal fibers tangentially into the protocerebral bridge. One group of 4–7 T5 neurons has cell bodies clustered laterally from the bridge, the second group of 8 T6 somata lies posteriorly from the tips of the bridge. In the bridge, immunostained fibers cross the brain midline and give rise to fine beaded terminals. Scale bars = 100 μm .

Figure 4.

Octopamine immunostaining in the central complex of the desert locust. (A) Frontal section showing octopamine immunoreactivity in the lateral accessory lobes (LAL) and anterior aspects of the central body. The lower division of the central body (CBL) and layer Ia of the upper division of the central body (I) are sparsely invaded by immunostained fibers. More prominent staining is present in the lateral accessory lobes (LAL), especially in the median olive (MO) and parts of the lateral triangle (LT). (B) The anterior lip (AnL) and the anterior bundles (AB) are free of immunostaining. (C) The lower units of the noduli (NL) and layers I and II of their upper units (I/II) are invaded by a sparse meshwork of immunolabeled processes. CBU, upper division of the central body. (D) Immunostained fibers from O1 neurons running along the dorsal face of the central body (arrowheads) give rise to numerous beaded processes extending into dorsal layers of the CBU. NU, upper unit of a nodulus. (E) The protocerebral bridge (PB, superimposed images from two adjacent sections) shows fine granular immunostaining originating from O2 fibers entering the bridge from its ventral side. In contrast to tyramine immunostaining, fibers connecting the two hemispheres of the bridge are not labeled. Scale bars = 100 μ m in A,B,D,E; 50 μ m in B.

Figure 5.

Partial reconstructions of octopamine-immunoreactive neurons innervating the central complex. (A) Pairs of ascending O1 fibers from the ventral nerve cord extensively innervate the median protocerebrum. Axonal processes run along the brain midline, turn laterally along the dorsal face of the lateral accessory lobes (LAL) and continue dorsally along the pedunculi of the mushroom bodies. Near the lateral edges of the upper division of the central body (CBU), pairs of collaterals branch off, run along the dorsal face of the central body and send numerous beaded processes into layers I and II of the CBU. A few processes continue into the noduli (No). Oe, oesophageal foramen. (B) Immunostaining in the protocerebral bridge (PB) could be traced to a bilateral pair of octopamine-positive O2 neurons with somata near the oesophageal foramen (group OA1/TA of Kononenko et al., 2009). From a common fascicle of primary neurites of these neurons, a single fiber projects dorsally through the posterior protocerebrum. It bifurcates into a collateral projecting toward the optic lobe and a medially projecting fiber. This one again bifurcates, and the two collaterals, one innervating the ipsilateral hemisphere of the bridge and the second, the contralateral hemisphere of the bridge, are joined by processes from the contralateral counterpart neuron. Scale bars = 100 μ m.

Figure 6.

Tyrosine-decarboxylase (TDC) immunostaining in the central complex of the desert locust. All neuropil areas showed some level of immunoreactivity, possibly related to synaptic epitopes. In addition, particular neuronal fiber systems were labeled more intensely. (A) Frontal section through the central body and lateral accessory lobes (LAL). Pairs of immunostained fibers run along the dorsal face of the central body (arrowheads) and into dorsal aspects of the lateral accessory lobes (double arrowheads). Prominently stained fibers pass through the median accessory lobe (arrows). CBU, CBL, upper, resp. lower division of the central body; P, pedunculus. (B) The anterior lip (AnL) shows immunostaining in a dense plexus of fibrous processes. mL, medial lobe of the mushroom body. (C) Bilateral clusters of neurons near the oesophageal foramen (Oe). Small somata (arrowheads) are those of T4 neurons innervating the noduli. (D) Frontal section through the noduli. Four pairs of fibers innervate the noduli and give rise to dense immunostaining in the lower units (NL) and coarser, granular staining in layers I and II (I, II) of the upper units. (E) Frontal section through the central body, posterior to the plane shown in A. Arrowheads point to immunostained fibers running along the dorsal face of the CBU. (F) Montage of four consecutive sections showing TDC immunostaining in the protocerebral bridge (PB). Fibers enter the two hemispheres of the bridge along their ventral sides (arrowheads). In addition, fiber fascicles of tangential neurons enter the PB at their tips (arrows) and connect the two hemispheres of the bridge through the midline. Scale bars = 100 μ m in A,B,C,E,F; 50 μ m in D.

Figure 7.

Tyramine β -hydroxylase (TBH) immunostaining in the central complex, frontal sections. (A) Section through the central body and lateral accessory lobes (LAL). Beaded processes are concentrated in layer Ia (Ia) of the upper division of the central body. In the LAL, the median olive (MO) and lateral triangle (LT) show enhanced staining. (B) The anterior lip (AnL) in front of the central body and the anterior bundles (AB) are largely free of immunostaining. (C) Bilateral clusters of large TBH-immunostained neurons near the oesophageal foramen (Oe). (D) In the noduli, the lower units (NL) and layer II of the upper units (II) are sparsely invaded by beaded immunostained processes. (E) Beaded processes from neurites running along the dorsal face of the central body extend into dorsal layers of the central body, particularly layers Ia and IIa of the upper division of the central body (Ia, IIa). (F) The protocerebral bridge is supplied from stained fibers entering the bridge along its ventral side (arrowheads). Beaded processes extend throughout the bridge, but do not cross the brain midline. Scale bars = 100 μ m in A,B,C,E,F; 50 μ m in D.

Figure 8. Partial reconstruction of TBH-immunostained innervations of the protocerebral bridge (PB). Two bilateral pairs of neurites, strikingly similar to octopamine-positive O2 fibers, give rise to beaded terminals throughout the bridge. Scale bar = 100 μ m.

Figure 9. Locations of cell bodies and principal fiber trajectories of immunostained neurons innervating the central complex of the desert locust. (A) Tyramine- and TDC-immunostained T1–T3, T4 and T5/T6 neurons, (B) octopamine-, TDC-, and TBH-stained O1 and O2 neurons. No, noduli; all other brain structures labeled as in Figure 1.

Table 1

Primary antibodies used

Antigen	Immunogen	Manufacturer	Dilution used
Octopamine	Octopamine-thyroglobuline conjugates	Rabbit polyclonal; Spörhase-Eichmann et al., 1992	1:5,000
Octopamine	Octopamine-thyroglobuline conjugates	Rabbit polyclonal; Eckert et al., 1992	1:5,000
Tyramine	p-Tyramine-glutaraldehyde-N- α -acetyl-L-lysine-N-methylamide	Rabbit polyclonal, Millipore, AB124	1:4,000
Tyrosine decarboxylase (TDC-1A)	Fusion protein of glutathione-S-transferase and <i>Caenorhabditis elegans</i> TDC-1A (aa 81–361)	Rabbit polyclonal; H. Robert Horvitz, Melissa Hunter-Ensor and Mark J. Alkema, Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA, USA	1:50 – 1:200
Tyramine β -hydroxylase (TBH)	<i>Drosophila melanogaster</i> TBH (aa 103–553)	Rat polyclonal; Monastirioti et al., 1996	1:300