Tyrosine hydroxylase immunostaining in the central complex of dicondylian insects

Josephine Timm | Mara Scherner | Jannik Matschke | Martina Kern | Uwe Homberg

Abstract
Dopamine acts as a neurohormone and neurotransmitter in the insect nervous system and controls a variety of physiological processes. Dopaminergic neurons also innervate the central complex (CX), a multisensory center of the insect brain involved in sky compass navigation, goal-directed locomotion and sleep control. To infer a possible influence of evolutionary history and lifestyle on the neurochemical architecture of the CX, we have studied the distribution of neurons immunoreactive to tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine biosynthesis. Analysis of representatives from 12 insect orders ranging from firebrats to flies revealed high conservation of immunolabeled neurons. One type of TH-immunoreactive neuron was found in all species studied. The neurons have somata in the pars intercerebralis, arborizations in the lateral accessory lobes, and axonal ramifications in the central body and noduli. In all pterygote species, a second type of tangential neuron of the upper division of the central body was TH-immunoreactive. The neurons have cell bodies near the calyces and arborizations in the superior protocerebrum. Both types of neuron showed species-specific variations in cell number and in the innervated areas outside and inside the CX. Additional neurons were found in only two taxa: one type of columnar neuron showed TH immunostaining in the water strider Gerris lacustris, but not in other Heteroptera, and a tritocerebral neuron innervating the protocerebral bridge was immunolabeled in Diptera. The data show largely taxon-specific variations of a common ground pattern of putatively dopaminergic neurons that may be commonly involved in state-dependent modulation of CX function.

KEYWORDS
dopamine, immunocytochemistry, insect brain, insect phylogeny, neuroanatomy, RRID: AB_2338690, RRID: AB_572268

1 | INTRODUCTION
Biogenic amines like dopamine, serotonin, histamine, and others control a variety of physiological processes in vertebrates and invertebrates. In mammals, the catecholamine dopamine plays key roles in cognitive abilities, motor coordination, modulation of sensory perception, learning, and memory (Smeets & González, 2000; Spanagel & Weiss, 1999). Perturbations of the dopamine system in
humans lead to brain disorders like Parkinson’s disease, schizophrenia, drug addiction, and mood disorders (Frith & Dolan, 1998; Rangel-Barajas et al., 2015). During vertebrate evolution, certain dopaminergic systems of the brain were highly conserved (Ryczko et al., 2016; Smeets & González, 2000; Yamamoto & Vernier, 2011), while others have newly emerged or have been lost secondarily (Yamamoto & Vernier, 2011).

Dopamine is also widely distributed in the brain of insects. Immunostaining for dopamine and/or tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis, revealed small numbers of neurons with processes that innervate most brain areas (honeybee: Schürmann et al., 1989; Schäfer & Rehder, 1989; blowfly, vinegar fly: Nässel & Elekes, 1992; Mao & Davis, 2009; desert locust: Wendt & Homberg, 1992; American cockroach: Hamanaka et al., 2016). Corresponding to its wide distribution, different functions have been demonstrated for dopamine in the insect nervous system, including a role in learning and memory, arousal, aggressiveness, and sleep–wake regulation (reviewed in van Swinderen & Andretic, 2011; Mustard et al., 2012; Yamamoto & Seto, 2014; Verlinden, 2018; Siju et al., 2021). Studies in the fly Drosophila, the honeybee, and the field cricket show that dopaminergic neurons are involved in associative learning (Das et al., 2016; Waddell, 2013; Wright, 2011). In all three species, dopamine mediates the reinforcement signal in aversive olfactory conditioning, but in Drosophila, dopamine also represents appetitive reinforcement (Huetteroth et al., 2015; Liu, Placais, et al., 2012; Waddell, 2013). In the fly, many types of dopaminergic neurons innervating certain parts of the mushroom body contribute to olfactory memory formation, and distinct sets of these neurons differentially regulate valence (positive or negative), olfactory memory strength and stability (Aso & Rubin, 2016; Boto et al., 2019; Huetteroth et al., 2015; Otto et al., 2020).

Evidence from the sphinx moth Manduca sexta, the honeybee, and Drosophila, in addition, suggests a role for dopamine in promoting motor activity and arousal (Claassen & Kammer, 1986; Harano et al., 2008; Kume et al., 2005; Pendleton et al., 2002; Riemensperger et al., 2011). Experimentally induced increases in the levels of dopamine or dopamine agonists led to increased locomotor activity, while injections of dopamine antagonists or depletion of dopamine in flies without active TH decreased arousal state and motor activity. Parts of these effects appear to be attributed to a role of dopamine and TH in sleep homeostasis and rest–activity rhythms, revealed in Drosophila (Dubowy & Sehgal, 2017; Helfrich-Förster, 2018; Liang et al., 2019; Potdar & Sheeba, 2018). Two types of dopaminergic neurons of the central complex (CX) are involved in promoting wakefulness and locomotor activity. A bilateral pair of dopaminergic neurons, termed PPL1, with projections to the fan-shaped body (FB), a subdivision of the CX, promotes wakefulness and arousal (Liu, Liu, et al., 2012; Ueno et al., 2012). The neurons are part of a neural circuit regulating sleep homeostasis (Donlea, 2017; Pimentel et al., 2016). Two different dopaminergic neurons, termed PPM3-EB, with arborizations in the ellipsoid body (EB) of the CX, on the other hand, promote wakefulness and locomotor activity under the control of the circadian clock in the fly brain (Liang et al., 2019). Therefore, promotion of motor activity is mediated by dopaminergic neurons of the CX in two different ways.

The CX is a midline-spanning system of neuropils common to all insect species. It consists of four subunits, the protocerebral bridge (PB), the upper (CBU), and lower (CBL) divisions of the central body, also termed FB, and EB, respectively, and a pair of globular-shaped noduli (Pfeiffer & Homberg, 2014). Three major categories of neurons innervating the CX have been distinguished across different species: Tangential neurons innervate distinct layers within the CX and largely provide input to the CX, while columnar and pontine neurons partition the CX into 16–18 columns or segments, provide internal right–left integration between columns and subunits, and, in the case of columnar neurons, send output signals to adjacent brain areas (Hanesch et al., 1989; Hulse et al., 2020; Pfeiffer & Homberg, 2014; Wolff et al., 2015). Evidence from several species including Drosophila, desert locust, cockroach, and others indicates that the columnar organization constitutes a neural network signaling head direction and spatial orientation, based on sky compass signals, visual panorama, and internally generated cues (Heinze & Homberg, 2007; Pisokas et al., 2020; Seelig & Jayaraman, 2015; Varga & Ritzmann, 2016; Zittrell et al., 2020). Outputs from the CX control heading and steering maneuvers during flight (Shiozaki et al., 2020) and walking (Green et al., 2019; Martin et al., 2015; Triphan et al., 2010).

Dopamine- and TH-immunoreactive neurons of the CX described in the cockroach Periplaneta americana (Hamanaka et al., 2016), the honeybee Apis mellifera (Schäfer & Rehder, 1989; Schürmann et al., 1989; Tedjakumala et al., 2017), the desert locust Schistocerca gregaria (Wendt & Homberg, 1992), and the flies Calliphora erythrocephala (Nässel & Elekes, 1992) and Drosophila melanogaster (Mao & Davis, 2009; Nässel & Elekes, 1992) share commonalities but also show differences. Neurons similar to the PPM3 dopaminergic neurons of flies have also been identified in the locust and cockroach (termed DP2 cells), and in the honeybee (termed Sp or C3b), while PPL1 neurons of flies are similar to DC2 and DC1 neurons in the locust, C3 neurons in the bee, and DCa neurons in the cockroach. In the honeybee and the two flies, additional cell types were described innervating the PB (flies) and central body (bee) that seem to have no counterparts in the locust and cockroach. To further uncover the role of dopamine in the insect CX, we have investigated the innervation of the CX by TH-immunoreactive neurons in a broad range of insect species covering most major dicondylian taxa. These range from firebrats to flies and are characterized by mandibles attached via two hinges (dicondyly) to the head capsule. The data show that a core pattern of dopamine innervation is highly conserved during insect evolution, but also reveal differences in cell numbers, innervated areas outside and inside the CX, and additional types of neuron only present in certain taxa.

2 | MATERIALS AND METHODS

2.1 | Insect species

Twenty species from twelve insect orders were used for the experiments. Firebrats (Thermobia domestica), house crickets (Acheta
domesticus), desert locusts (S. gregaria), Annam walking sticks (Medauroida extradentata), Madeira cockroaches (Rhyparobia maderae), assassin bugs (Platymerys biguttatus), honeybees (A. mellifera), mealworm beetles (Tenebrio molitor), red flour beetles (Tribolium castaneum), and blowflies (C. erythrocephala) were obtained from insect cultures at Philipps-Universität Marburg (Department of Biology, Animal Physiology). Firebrats, mealworm beetles and flies were kept at 25°C. House crickets were kept at a temperature of 22°C and a humidity of 50%. Desert locusts were kept at 28°C. The cockroaches were kept in a plastic box at a constant temperature of 24°C and a humidity of 50%. The light–dark rhythm was 12:12 h for all six species. Stick insects were kept at room temperature in a terrarium with blackberry branches. Dr Andreas Brune (Max-Planck Institute for Terrestrial Microbiology, Marburg) provided blind workers of the Asian subterranean termite Coptotermes gestroi. Backswimmers (Notonecta glauca), water striders (Gerris lacustris), common whirligig beetles (Gyrinus substriatus), and larvae of the dragonfly Anax imperator and the damselfly Chalcolestes viridis were caught in ponds near the Department of Biology. Mottled shield bugs (Rhaphigaster nebulosa) were collected in the department's stairwell in spring. Sphinx moths (M. sexta) were obtained from Dr Monika Stengl (University of Kassel). Vinegar flies (D. melanogaster) were provided by Dr Christian Bökel (Department of Biology, Philipps-Universität Marburg). Praying mantises (Hierodula membranacea) were obtained from a commercial vendor.

2.2 | Immunocytochemistry of TH

Animals were cold anesthetized in the refrigerator. Brains were dissected on ice in phosphate-buffered saline (PBS, 0.1 mol l⁻¹). For small brains (water striders, termites, vinegar flies, shield bugs), a lower molarity (PBS, 0.01 mol l⁻¹) was used throughout the staining protocol. Brains were submerged for 30 min at 4°C in 2% paraformaldehyde fixative (PFA) in Millonig's phosphate buffer (0.13 mol l⁻¹ NaH₂PO₄ × H₂O, 0.1 mol l⁻¹ NaOH, 0.3 mmol l⁻¹ CaCl₂, 1.2% glucose, pH 7.3–7.4) before fixing for another 30 min at room temperature followed by washing 4 × 10 min in 0.1 mol l⁻¹ PBS.

Brains were preincubated overnight at 4°C in 0.1 mol l⁻¹ PBS containing 0.3% Triton X-100 (TrX), 2% bovine serum albumin, and 2% normal goat serum (NGS) to reduce nonspecific staining. Following, the brains were incubated in monoclonal TH antibody (Immunostar, cat# 22941, RRID: AB_572268), diluted at 1:1000 in 0.1 mol l⁻¹ PBS, 0.3% TrX, and 2% NGS, for at least 4 days at 4°C. After washing in 0.1 mol l⁻¹ PBS and 0.3% TrX for 4 × 15 min, incubation in Cy3-conjugated goat antimouse (GAM) (1:300) (Jackson ImmunoResearch, cat# 115-165-146, RRID: AB_2338690) and Alexa Fluor Phalloidin 488 (Alexafluor488) (1:200) (Thermo Fisher), diluted in 0.1 mol l⁻¹ PBS, 0.3% TrX, and 2% NGS, was carried out for at least 3 days at 4°C. Prior to embedding in Permount, brains were dehydrated through an ascending series of ethanol solutions and clarified in an ethanol-methyl salicylate solution (50:50) followed by pure methyl salicylate for at least 30 min.

In addition to wholemounts, brains of the sphinx moth M. sexta were sectioned using a vibrating blade microtome (VT1200S, Leica, Wetzlar, Germany). After incubation with Cy3-GAM, brains were washed in 0.1 mol l⁻¹ PBS and 0.3% TrX for 3 × 15 min and fixed in 4% PFA in sodium phosphate buffer (NaPi) for 1 h. After briefly washing in 0.1 mol l⁻¹ PBS and 0.3% TrX, the brains were embedded and aligned in preheated gelatin/albumin. After hardening, the gelatin block was fixed in 8% formaldehyde at 4°C overnight. Brains were sectioned at 200 μm. The sections were embedded in Permount as described above.

2.3 | Immunoblotting

A protocol modified from Utz et al. (2008) and Lawrence and Besir (2009) was used. Brains from selected insects (A. domesticus, N. glauca, G. lacustris) were dissected in 0.01 mol l⁻¹ PBS with protease inhibitors (Complete protease inhibitor cocktail, Roche Diagnostics) and homogenized before performing gel electrophoresis in 10% polyacrylamide gel. PageRuler prestained protein ladder (Thermo Scientific) was used as molecular weight marker. After electrophoresis, the proteins were blotted on an OptiTiran BA-S 85 membrane (Whatman). The membrane was blocked for 2 h in 5% SlimFast (Slim Fast Foods, Englewood, NJ) in PBS and 0.1% Tween 20 before incubated overnight in anti-TH diluted at 1:1000 in PBS, 2.5% SlimFast, and 0.1% Tween 20. After washing 4 × 10 min in PBS, 0.05% Tween 20, 1% TrX, and 0.1% sodium dodecyl sulfate (SDS), the membrane was incubated for 2 h in HRP-conjugated GAM (Jackson ImmunoResearch, Cat# 115-035-003), diluted at 1:5000 in PBS and 0.1% Tween 20. The membrane was rinsed three times and washed 2 × 15 min in PBS, 0.05% Tween 20, 1% TrX, and 0.1% SDS before using the SuperSignal West Pico chemiluminescent substrate kit (Pierce) for chemiluminescence reaction.

2.4 | Antibody characterization

The TH antibody (Immunostar, Cat# 22941, RRID: AB_572268) is a monoclonal antibody raised in mouse against rat TH. The antibody was made against full length TH purified from rat P12 cells and recognizes an epitope in the catalytic core of TH. The antibody has been used to recognize TH, which is highly conserved throughout evolution (Calvo et al., 2011; Neckameyer & Quinn, 1989), across a wide range of animals, including insects (Hartenstein et al., 2017; Tedjakumala et al., 2017). In Western blots of protein homogenates from HEK293 cells transfected with human TH, the antibody detects a protein band at 60 kDa (Immunostar), which corresponds to the molecular weight of TH in Drosophila (58 kDa; Neckameyer & Quinn, 1989), cockroach P. americana and the cricket Gryllus bimaculatus (66 kDa; Hamanaka et al., 2016). We performed immunoblotting of TH from three selected species. The antibody detected a single lane at 54 kDa in the water strider G. lacustris, at 58 kDa in the cricket A. domesticus, and at 62 kDa in the backswimmer N. glauca (Figure 1).
2.5 | Image acquisition and reconstructions

Sections and wholemounts were scanned with a confocal laser scanning microscope (TCS SP5, Leica Microsystems). All scans were carried out with an image resolution of 1024 × 1024. The z-step size was 1.51 μm. For overview scans, a ×10 oil immersion objective (HC PL APO ×10/0.40 IMM CS2) was used with a speed of 400 Hz and a line average of 1. A ×20 oil immersion objective (HC PL APO ×20/0.75 Imm Corr CS2) was used for detailed scans with a speed of 200 Hz and a line average of 2. Small brains were scanned with a ×63 oil immersion objective (HCX PL APO ×63/1.40 oil PH3). Structures labeled with Alexafluor488 were detected with an argon laser (488 nm) and structures labeled with Cy3 with a DPSS-laser (561 nm).

All data stacks were evaluated with Amira 6.5 (Thermo Fisher Scientific, Waltham, MA). In large brains, the left and right brain hemispheres had to be scanned individually. The two data stacks were aligned and merged. For reconstructions the neurons were manually marked based on the Cy3 staining. Therefore, a LabelField was created using the Create → Data function and was connected to the data stack. The neurons were marked and added to the Material using the Segmentation Editor. An Arithmetic module was created in the Project View. The data stack was assigned as input A and the LabelField as input B. In order to hide the areas not assigned to the Material, the formula A*(B > 0) was used as expression in the settings of the Arithmetic module. This resulted in a data stack only containing the marked neurons. By attaching the Voltex function, it could be visualized three-dimensionally or displayed as a maximum projection using the Image Ortho Projection function. Neuropils were reconstructed based on the Alexafluor488 staining. Captions were added using Adobe Photoshop.

3 | RESULTS

Across all insect species similar types of CX neurons showed TH immunolabeling. One cluster of neurons contributing to innervation of the CX is located laterally from the calyces of the mushroom bodies. Fibers from some of these neurons enter the central body from its anterior surface or between the CBL and CBU. These neurons are most likely homologous to the dopamine-immunolabeled DC1 and DC2 neurons in the locust (Wendt & Homberg, 1992) and PPL1 neurons of flies (Nässel & Elekes, 1992) and will be referred to as DC neurons throughout this study. A second cluster of CX neurons has cell bodies near the lateral tips of the PB. These neurons project first to the lateral accessory lobe before their axonal fibers enter the central body from its ventral side. The neurons are strikingly similar to dopamine-immunolabeled DP2 neurons in the cockroach (Hamanaka et al., 2016) and locust (Wendt & Homberg, 1992) and to PPM3 neurons in flies (Nässel & Elekes, 1992). These neurons will be termed DP2 neurons.

3.1 | TH immunostaining in the CX of the firebrat (Zygentoma)

In the brain of the firebrat T. domestica, six to eight bilateral pairs of TH-immunolabeled DP2 neurons innervate the central body (Figure 2). Their cell bodies are loosely clustered in the posterior pars intercerebralis near the lateral ends of the PB. Cell body fibers fasciculate, bypass the central body laterally and enter the lateral accessory lobe dorsolaterally. Neurons give rise to ramifications in the lateral accessory lobe (Figure 2). Their main fibers make a sharp turn toward the brain midline, enter the central body ventrally, and give rise to fan-like arborizations in the central body. The upper division of the central body (CBU) is innervated densely by TH-immunoreactive processes, the lower division of the central body is largely free of immunostaining (CBL) (Figure 2(a–c)). The PB does not show TH immunostaining, noduli could not be identified. Bilateral clusters of 9–11 DC-type neurons with cell bodies lateral to the calyces are also immunostained (Figure 2(d)). The neurons send fibers ventromedially and partly across the brain midline in commissures anterior and ventral from the central body, but do not innervate the central body. Instead, the neurons innervate parts of the trauben (Figure 2(e)), globular protrusions of the medial lobes of the mushroom body that are targeted by DC neurons also in other species (e.g., locust DC1 neurons: Wendt & Homberg, 1992; fly PPL1 neurons: Mao & Davis, 2009).

3.2 | TH immunostaining in the CX of Odonata

Two dragonfly species were examined as larvae, the willow emerald damselfly (C. viridiss, Lestidae) and the emperor dragonfly (A. imperator,
Aeshnida). Both species showed very similar branching patterns of TH-immunoreactive neurons (Figure 3). Based on differential staining, three layers were distinguished in the CBU, a superior layer, termed CBU-I, showing sparse immunostaining, an intermediate layer, CBU-II, which was devoid of immunoreactivity, and an inferior, most densely labeled layer, termed CBU-III (Figure 3(c,d,f)). In both species two clusters of tangential neurons were identified that send processes into the central body. The upper division of the central body (CBU) is densely supplied by immunoreactive processes, while the lower division of the central body (CBL) is largely free of immunostaining. No immunostaining was found in the PB. (b,c) Single optical sections through the central body and LALs at an anterior (b) and more posterior level (c). TH immunostaining in (b,d,e) is shown in magenta, phalloidin fluorescence in green. Arrows in (b,c) point to hairpin turns of immunostained fibers from DP2 neurons. (d) Stack of 40 optical sections showing immunolabeled DC neurons with cell bodies lateral to the calyces (CA, arrowheads). The neurons send fibers medioventrally, with some processes crossing the brain midline anterior to the central body (arrows), but do not invade the central body. (e) Stack of 10 optical sections. The DC-type neurons have ramifications in certain parts of the trauhen (TRA, arrowheads), swollen protrusions of the medial lobes of the mushroom body. Scale bars = 30 μm [Color figure can be viewed at wileyonlinelibrary.com]
FIGURE 3  Tyrosine hydroxylase (TH) immunostaining in the central complex of dragonfly larvae. (a–e) Emperor dragonfly, Anax imperator. (a) Maximum intensity visualization of 72 optical slices illustrating the somata of DC-type neurons lateral to the calyces (CA; yellow circles) that innervate the central body (CB, outlined in red) frontally. (b) Single optical section showing wide arborizations of these neurons in the superior medial protocerebrum (SMP). Neurites continue via the anterior bundle (AB) toward the central body, but most fibers bypass the central body. A few processes cross the brain midline along the frontal face (yellow arrow) of the central body (outlined in red) and innervate layer I (CBU-I) of the upper division of the central body (c,d). White arrows show fiber fascicles of DP2-type neurons. (c,d) Single optical sections through the central body at a more anterior (c) and more posterior (d) level. Two layers of the CBU and the CBL are immunoreactive. The dorsal layer of the CBU (CBU-I) and the CBL are innervated only sparsely. The ventral layer (CBU-III) is densely innervated. (e) Frontal reconstruction of DP2-type TH-immunostained neurons innervating the lateral accessory lobes (LAL) and layer III of the CBU. (f–h) TH immunostaining (magenta) and phalloidin fluorescence (green) in the willow emerald damselfly, Chalcolestes viridis. (f) Maximum intensity visualization of 28 optical sections illustrating TH immunostaining in the CBU of C. viridis. Layer I of the CBU (CBU-I) shows sparse immunostaining while layer III (CBU-III) is densely innervated by DP2 neurons. Their fibers (white arrows) have fine ramifications in the lateral accessory lobe (LAL) before entering CBU-III. Yellow arrow points at fibers of DC neurons in the anterior bundle. (g) Some fibers of DP2 neurons have side branches in the PB; stack of 10 optical sections. (h) Stack of four optical slices illustrating TH-immunostained processes in a middle layer of the noduli (NO). L, lateral. Scale bars = 50 μm (a–d), 100 μm (e,f), and 20 μm (g,h) [Color figure can be viewed at wileyonlinelibrary.com]
immunoreactivity (Figure 3(c–f)) while the CBL is innervated only sparsely (Figure 3(c)). A few side branches from the fibers in the w-bundle, in addition, innervate the inferior clamp and the lateral ends of the PB (Figure 3(g)). This staining pattern closely resembles the general morphology of DP2 neurons in flies and locusts. In desert locusts, two DP2 neurons, termed TL5, invade the PB and the CBL, consistent with the sparse labeling of both neuropils in the dragonflies.

3.3 | TH immunostaining in the CX of the desert locust and house cricket (Orthoptera)

Immunostaining for TH was studied in two orthopteran species, the desert locust S. gregaria (Acrididae) and the house cricket A. domesticus (Ensifera) (Figure 4). In both species, two groups of TH-immunoreactive tangential neurons, DP2-and DC neurons, were identified that arborize in the CX. In the cricket, the CBU can be divided into three layers, an anterior-dorsal layer (CBU-I), an intermediate layer (CBU-II), and a ventral layer (CBU-III). Layer II and posterior parts of layer I (CBU-Ib) are subdivided into eight cone-like domains (Figure 4(a,c–e)). Layer I (CBU-I) is innervated by fibers from a subpopulation of 18–20 DC-type neurons with cell bodies lateral to the calyces. Their fibers pass by the central body along its anterior face. Side branches innervate layer I with uniformly distributed beaded endings (Figure 4(c,d)). DP2-type neurons innervate both the CBU and the CBL. Their cell bodies (27–30 per hemisphere) are located in the posterior pars intercerebralis (Figure 4(a,b)). Cell body fibers project to a fiber plexus in the upper part of the lateral accessory lobe (Figure 4(a)). From here, immunostained processes invade the lateral accessory lobe; others project dorsally along the anterior face of the central body and in a fascicle along the medial lobe of the mushroom body toward the superior protocerebrum (asterisk in Figure 4(a)). A forth bundle of immunolabeled processes projects medially and innervates the CBL and CBU from ventral direction (Figure 4(a–c)). Four fascicles of side branches from these fibers innervate the CBL (Figure 4(c)). In addition, two layers of the CBU (CBU-II and CBU-III) are densely supplied by stained processes. Layer III shows immunolabeling in a patchy pattern (Figure 4(d,e)), and four fascicles of fibers, each of which divides again, gives rise to dense labeling in the eighth domains of CBU-II (Figure 4(d)). The lower units of the noduli and a dorsal layer of the upper units of the noduli are sparsely supplied by immunoreactive endings (Figure 4(e,f)). The PB shows no immunoreactivity.

The CX of the desert locust is innervated by DC- and DP2 neurons in a pattern virtually identical to that reported for dopamine immunostaining (Wendt & Homberg, 1992). About 54 bilateral pairs of DP2 neurons with cell bodies located in the posterior pars intercerebralis innervate the PB (Figure 4(g)), the CBU, CBL, and the lateral accessory lobe (Figure 4(h,i)). Owing to the large number of immunolabeled DP2 neurons, the lateral accessory lobes and the CBU show particularly strong and dense immunostaining, while labeling in the PB and CBL is less dense. About 24 bilateral pairs of somata were counted in the DC cell cluster lateral from the calyces (Figure 4(i)). While most fibers from this cluster innervate the lobes of the mushroom body, some fibers passing anteriorly along the central body or entering the ventral groove between CBL and CBU send side branches into layer I of the CBU, which in a superficial dorsal strip is more faintly stained than the rest of the CBU. The dorsal layer I of the upper units of the noduli shows sparse labeling, likely from DP2 neurons (Figure 4(i)).

3.4 | TH immunostaining in the CX of the Annam walking stick (Phasmatodea)

Similar to the cricket and the locust, the CX of the stick insect M. extradentata is innervated by two types of TH-immunoreactive tangential neurons, DC neurons and DP2 neurons (Figure 5). Both clusters are located in the posterior protocerebrum. From the posterior pars intercerebralis, fibers from about 25 bilateral pairs of DP2 neurons project anteriorly and enter the lateral accessory lobes (Figure 5(a,b)). From here, a bundle of processes extends along the mushroom body lobes toward the superior protocerebrum (arrows in Figure 5(a)), another fiber fascicle medially to the central body. The neurons innervate layers II and III of the CBU with immunostaining being concentrated in eight wedge-shaped (CBU-II) resp. spheroidal domains (CBU-III) (Figure 5(a,d)). The second TH-immunoreactive cell group, DC neurons lateral from the calyx, consists of 13–15 neurons in each hemisphere (Figure 5(b)). Their fibers pass by the vertical lobes of the mushroom bodies. Many neurons innervate the lobes of the mushroom body, but some fibers, projecting along the anterior face of the central body, give off side branches into the anterior dorsal layer of the CBU (CBU-I, Figure 5(c)). An intermediate layer of the noduli is sparsely innervated, apparently from DP2 neurons, but the PB and the CBL are free of staining (Figure 5(c,e)).

3.5 | TH immunostaining in the CX of Dictyoptera

Immunostaining was studied in the praying mantis, H. membranacea, the Madeira cockroach, R. maderae, and the Asian subterranean termite, C. gestroi (Figure 6). In the praying mantis, as in most other species studied, DC- and DP2-type immunoreactive tangential neurons innervate the CX. About 45 bilateral pairs of DP2 neurons with somata near the ends of the PB innervate the CBU from ventral direction (Figure 6(a–c)). The neurons have wide ramifications in the lateral accessory lobes. Their processes pass in 10 fiber fascicles through the CBL and innervate the noduli, two ventral layers of the CBU (CBU-II and CBU-Ib) with beaded terminals and 10 cone-like columns in a middle layer (CBU-Ia). The CBL is free of immunoreactive terminals. In addition, fibers from a subset of about 26 DC neurons per hemisphere with cell bodies lateral to the calyces innervate the most anterior-dorsal layer of the CBU (CBU-I) as their fibers cross the brain midline in front of the central body. Immunostaining is concentrated in 10 cone-like domains of layer I (Figure 6(a)). The PB is free of immunoreactive fibers.

In the Madeira cockroach, about 32 DP2-type neurons per hemisphere with cell bodies in the pars intercerebralis are TH-immunoreactive (arrowheads in Figure 6). The neurons send cell body fibers to the lateral
Figure 4  Tyrosine hydroxylase (TH) immunostaining in the central complex of Orthoptera. (a–f) House cricket, Acheta domesticus, (g–i) desert locust, Schistocerca gregaria. (a,b) Reconstruction of DP2-type TH-immunoreactive neurons in frontal (a) and sagittal (b) views. From cell bodies clustered near the lateral ends of the protocerebral bridge (PB), fibers enter a plexus of immunolabeled processes in a dorsomedial region of the lateral accessory lobe (LAL). From here, processes extend into the LAL, into areas lateral from the central body, and along the vertical lobe of the mushroom body toward the superior protocerebrum (asterisks). A major fiber bundle projects medially and innervates the upper and lower divisions of the central body (CBU, CBL) from their ventral sides. (c–e) Single optical sections through the central body from anterior (c) to posterior (e). An anterior dorsal layer of the CBU (CBU-la) shows uniform granular immunolabeling originating from DC-type neurons, which becomes more sparse toward posterior levels (CBU-Ib). The intermediate and ventral layer of the CBU (CBU-II, CBU-III) and the CBL show prominent immunostaining originating from DP2 neurons (see a). Staining in CBU-II is organized in eight cone-like domains; immunolabeling of layer III (CBU-III) has a patchy appearance. The CBL is innervated from ventral by four fiber bundles. (e,f) The lower units of the noduli (NOL) and a dorsalmost layer of the upper units of the noduli (NOU) show sparse immunostaining, originating from DP2 neurons. (g) Maximal projection of the PB of the desert locust. It is innervated evenly by immunoreactive fibers from DP2 neurons. (h) Strong immunoreactivity throughout the CBU, except for a narrow dorsalmost layer (arrowheads) originates from DP2- and DC neurons. DP2 neurons massively invade the LALs. The CBL is innervated evenly. (i) DC neurons with somata lateral to the calyces (CA; arrows). The LAL, the NOU (arrowheads) and the PB show immunostaining. Double arrowheads point at fiber bundles of DP2 neurons. p, posterior; d, dorsal. Scale bars = 100 μm (a,g–i), 50 μm (b–e), and 30 μm (e,f)
accessory lobes; from here axonal processes invade the CBL and, via five fiber bundles, layer II of the CBU (Figure 6(e,f)). Staining in the CBL and CBU is concentrated in columnar domains (eight columns in the CBU). The noduli show sparse staining in two narrow layers, likely resulting from some of these neurons (inset in Figure 6(g)). Most fibers from DC neurons (about 32 neurons per hemisphere) with cell bodies lateral from the calyces (arrows in Figure 6(g)) innervate the lobes of the mushroom bodies (Figure 6(d,e)) but some fibers cross the brain midline along the dorsal face of the particularly prominent anterior lip and from here, send side branches into layer I of the CBU which shows sparse TH immunolabeling (Figure 6(d,e)).

Workers of the subterranean termite Coptotermes gestroi are blind, have no compound eyes, highly reduced optic lobes but large mushroom bodies with highly convoluted lobes. The cell bodies of about 32 bilateral pairs of TH-immunostained DP2 neurons are shifted ventrally owing to the large mushroom bodies. Based on TH immunostaining, three layers could be distinguished in the CBU, a strongly immunostained superior-dorsal layer, an intermediate layer (CBU-II) and a ventral layer (CBU-III). Eight cone-like columns in CBU-II as well as CBU-III are innervated by the DP2-type cells (Figure 6(h)). Their cell body fibers invade the lateral accessory lobes and then send axonal processes to the CBU and noduli. The CBL is largely free of immunoreactivity (Figure 6(i)). The dorsalmost layer of the CBU (CBU-I), invaded by DC neurons, has a homogeneous beaded appearance, while immunostaining in CBU-II and III, originating from DP2 neurons, is concentrated in eight columnar domains. The lower division of the central body (CBL) is free of immunostaining. (e) Confocal section showing immunoreactivity in a narrow median layer of the noduli (NO). The PB is free of immunostaining. Scale bars = 100 μm [Color figure can be viewed at wileyonlinelibrary.com]

3.6 | TH immunostaining in the CX of true bugs (Hemiptera)

Four heteropteran species were studied, the water strider G. lacustris, the backswimmer N. glauca, the assassin bug P. biguttatus, and the shield bug R. nebulosa (Figures 7 and 8). In G. lacustris, three types of TH-immunoreactive neurons innervate...
the CX, DC- and DP2-type tangential neurons and one type of columnar neuron. About 33 columnar neurons connecting the PB with the CBU and the noduli exhibit TH immunostaining (Figure 7 (a,b,e)). Based on projection pattern, these neurons are similar to CPU4/CPU5 neurons in locusts (Heinze & Homberg, 2008) and P-FN neurons in flies (Sullivan et al., 2019). Both the PB and the noduli are particularly large compared to other species. The columnar neurons send fasciculated cell body fibers into the PB (Figure 7(a,b)). From the PB, fibers project in four bilateral pairs of bundles, the w-, x-, y-, and z-bundles, through the posterior chiasma and innervate intermediate layers of the CBU (CBU-II) and noduli (Figure 7(b,e)). Judged from cell body counts, each column of the PB and CBU appears to be innervated by four of these neurons. Six to seven somata of DP2 neurons lie lateral to the cell

FIGURE 6  Legend on next page.
Tyrosine hydroxylase (TH) immunostaining in the central complex of Dictyoptera. (a–d), Tyrosine hydroxylase (TH) immunostaining in the central complex of Dictyoptera. (a–d). Their fibers join the w-bundles and innervate the lateral accessory lobes. Axonal fibers enter the central body posterior-ventrally and innervate layer II of the CBU, two layers in the CBL and the noduli (Figure 7(b–e)). Eight to fifteen DC neurons have cell bodies dorsolateral to the central body (Figure 7(a)). Some fibers from these neurons ramify in the superior medial protocerebrum, enter the CBU anteriorly, and innervate layer I of the CBU with beaded terminals (Figure 7(a,b,e)).

In the backswimmer N. glauca, 7 DP2 neurons and about 12 DC neurons showed TH immunostaining (Figure 7(f–i)). As in the water strider, the CBL of N. glauca has a distorted toroidal shape (Figure 7(h)). Fibers of DP2 neurons project anteroventrally and arborize in the lateral accessory lobes (Figure 7(f)). From here, axonal fibers project to the central body and give rise to eight fiber bundles that ascend posterior from the CBL and innervate a posterior ventral layer of the CBU (CBU-III).

Some neurons have also terminals in the CBL and the noduli (Figure 7(f–i)). DC neurons have cell bodies located lateral to the calyces. Some fibers of these neurons pass anteriorly from the central body across the brain midline and, along this course, send side branches into the anterior-dorsal layer I of the CBU (Figure 7(i)). The PB is not TH-immunoreactive.

In contrast to the compact shape of the central body in the backswimmer and water strider, the central body in the shield bug and assassin bug is elongated and slender (Figure 8). In the shield bug, only the CBU is innervated by TH-immunoreactive fibers. Staining is relatively sparse compared to other species and originates from about 8 DP2- and 10 DC-type neurons (Figure 8(a)). Fibers passing anteriorly along the central body, presumably from DC neurons, innervate an anterior layer of the CBU (CBU-I in Figure 8(b)), and DP2-type neurons a more posterior layer III. In contrast to all other species studied, the bulbs lateral from the central body show strong TH immunostaining (Figure 8(a)), but its neuronal origin could not be determined. Noduli could not be identified. In the assassin bug P. biguttatus, 12 bilateral pairs of DP2 neurons give rise to immunostaining in the lateral accessory lobes, the CBL, posterior ventral layers of the CBU (Figure 8(c,e)), and the noduli. A dorsal layer of the CBU (CBU-I) is innervated by side branches from fibers that cross the brain midline anteriorly from the central body (Figure 8(d)). These fibers are only weakly labeled. Whether they originate from a cluster of cell bodies lateral to the calyces (DC neurons) could not be determined. The PB is free of immunoreactive fibers.

3.7 | TH immunostaining in the CX of the honeybee (Hymenoptera)

One hymenopteran species, the honeybee A. mellifera, was examined. The CX of the bee is innervated by two types of TH-immunoreactive neurons, DC- and DP2 neurons (Figure 9). About 15 DP2 neurons with cell bodies in the posterior pars intercerebralis innervate the CBU, the CBL, and the noduli. The neurons have wide ramifications in the lateral accessory lobes (Figure 9(a,f)). Side branches enter the ishmu tract toward the brain midline and give rise to dense innervation of the CBU, layer III of the CBU and the noduli (Figure 9(a,b,d)). A ventral layer of the CBL is only sparsely supplied by immunoreactive profiles (Figure 9(d)). The noduli show strong immunostaining except for the small spheroidal subunits (Figure 9(e)), probably corresponding to the lower units of the noduli in other species. In addition, a large number of about 125 DC neurons per hemisphere with cell bodies ventral from the lateral calyx show TH immunoreactivity. Most of these neurons innervate the mushroom bodies, but a few fibers from these neurons cross the brain midline in front of the central body (Figure 9(f)) and send side branches into the anterior dorsal layer of the CBU (CBU-I). TH immunolabeling in CBU-I has a peculiar patchy appearance (Figure 8(c)). More posteriorly, a ventral layer, CBU-IIb is only weakly stained (not shown) while staining in dorsal aspects of the CBU (CBU-IIa) is sparse but distinctly beaded (Figure 9(d)). Whether staining in CBU-IIa originates from DP2- or DC cells could not be determined. The PB shows no immunoreactivity.

FIGURE 6 Tyrosine hydroxylase (TH) immunostaining in the central complex of Dictyoptera. (a–c) Praying mantis, Hierodula membranacea. (d–g) Madeira cockroach, Rhyparobia maderae. (h,i) Asian subterranean termite, Coptotermes gestroi. (a–c) Optical sections of the central body at an anterior (a), an intermediate (b), and a posterior (c, stack of three slices) level. TH immunostaining is shown in magenta, phalloidin fluorescence in green. Based on TH immunostaining, three layers can be distinguished in the upper division of the central body (CBU). The superior layer I (CBU-I) and an intermediate layer (CBU-IIa) are characterized by innervation of 10 cone-like columns from ventral direction. Two ventral layers, CBU-IIb and CBU-III, are more uniformly innervated by TH-immunolabeled processes. CBU-IIb in an irregular patchy fashion. The lower division of the central body (CBU) is largely free of immunostaining. The noduli (NO) show beaded immunolabeling. Arrows in (a) point to labeled neurites of DP2 neurons that connect the lateral accessory lobes (LAL) with layers II and III of the CBU. (d–f) Frontal images of TH staining in the central body of the Madeira cockroach at anterior (d, stack of 8 optical sections), intermediate (e, single section), and posterior levels (f, stack of 16 optical sections). (d) Fibers from DC neurons cross the brain midline near the anterior lip (ALI) and send side branches (arrows) into layer I of the CBU (CBU-I). (e,f) The CBL and cone-like domains in the CBU-II are innervated by processes from TH-immunostained DP2 neurons (arrows in f). (g) Stack of 98 optical sections showing the somata of DC neurons lateral to the calyces (CA, arrows) and DP2 neurons in the posterior pars intercerebralis (arrowheads). Inset shows two immunostained layers of the noduli (NO). (h) Stack of 17 optical sections illustrating TH immunostaining in the central body of the termite C. gestroi. An intermediate layer of the CBU (CBU-II) is innervated densely in cone-like columns. A thin dorsal layer (CBU-I) shows strong immunoreactivity. Labeled neurites connect the CBU and the lateral accessory lobes (LAL). The immunolabeled cell bodies dorsal from the central body are not connected to the central body. (i) The CBL is largely free of immunoreactivity. ML, medial lobe of the mushroom body; PED, pedunculus; VL, vertical lobe of the mushroom body. Scale bars = 100 μm (a–g), 50 μm (h, also applies to i) [Color figure can be viewed at wileyonlinelibrary.com]
3.8 TH immunostaining in the CX of beetles (Coleoptera)

Immunostaining was studied in the mealworm beetle *Tenebrio molitor*, the red flour beetle *T. castaneum*, and the whirligig beetle *G. substriatus* (Figure 10). In all three species, DC- and DP2-type neurons showed TH immunostaining. In *T. molitor*, one bilateral pair of DC neurons out of about 33 immunostained cells with somata lateral from the calyces (Figure 10(d)) innervate the dorsalmost layer I of the CBU and give rise to beaded terminals throughout CBU-I (Figure 10(a)). The ventralmost layer III of the CBU and the ventral parts of the noduli are instead invaded by about 11 DP2 neurons with cell bodies near the lateral ends of the PB (Figure 10(b,c)). These neurons have wide, presumably dendritic ramifications in the lateral accessory lobes (not
shown). The CBL and PB are free of immunostaining (Figure 10(a,d)).
The innervation of the CX of T. castaneum by TH-immunostained pro-
cesses is slightly different. In the CBU, three layers can be distin-
guished based on differences in TH immunoreactivity. The middle
layer shows weakest immunoreactivity (CBU-II). A dorsal layer (CBU-I)
is innervated frontally by processes from DC neurons with cell bodies
lateral to the calyces. Thirteen to fifteen DC neurons were counted
but only some of them invade the CBU. The CBL, a ventral layer of
the CBU (CBU-III), and the noduli are invaded by processes from
about 11 DP2 neurons per hemisphere. Like in T. molitor, these neu-
rons have fibers bypassing the central body laterally, invade the lateral
accessory lobe, and then send axons to the central body. The PB is
again free of TH-immunoreactive fibers.

TH-immunoreactive DC neurons (about 14 somata per hemisphere)
and DP2 neurons (about 10–11 somata) were also identified in G. sub-
striatus. Unlike in any other insect species studied, the DP2 neurons not
only innervate the lateral accessory lobe, but in addition send fine, pre-
sumably dendritic processes also to a small neuropil area in the posterior
superior protocerebrum in close apposition to the calyces of the mush-
room body (Figure 10(g)). The neurons invade CBU-III and, more sparsely,
the noduli. DC neurons with somata lateral from the calyces (Figure 10
(g) have ramifications in the superior medial protocerebrum and invade
the most dorsal layer of the CBU (CBU-I) with fine beaded terminals
(Figure 10(h,i)). The PB is free of immunoreactive fibers.

3.9 | TH immunostaining in the CX of the giant
sphinx moth (Lepidoptera)

In the sphinx moth M. sexta, DC- and DP2 neurons show TH immuno-
stimulating and innervating the CBL, the CBU, and parts of the noduli
(Figure 11). Because the neuraxis in the central brain of Lepidoptera is
not only bent upward like in Hymenoptera and other more basal taxa,
but even backward, horizontal sections of the brain were prepared
(Figure 11(c–e)) that more closely resemble frontal images of brain
structures in all hitherto described species. About 28 DC neurons with
cell bodies lateral to the calyces of the mushroom body were immuno-
stimulated. Their cell body fibers bypass the calyces dorsally and give rise
to wide ramifications in the superior medial protocerebrum (Figure 11
(a) that intermingle with processes from other labeled neurons. From
here, about five fibers per hemisphere continue medioventrally, cross
the brain midline in a commissure along the anterior dorsal face of the
central body and give off side branches into a superficial posterior
dorsal layer of the CBU (CBU-I in Figure 11(b)). In addition, bilateral
clusters of about 17 DP2 neurons with somata near the lateral tips of
the PB are TH-immunoreactive. Their cell body fibers project anteri-
only to the lateral accessory lobe, give off side branches into the lat-
eral accessory lobe, and then make a sharp turn medially (Figure 11(c,
d)). The neurons give rise to a second area of arborizations in neuropil
dorsal and posterior to central body, likely corresponding to the supe-
rior clamp in flies (arrowheads in Figure 11(c)) before their processes
invade the central body along its anterior face. The neurons innervate
an anterior and posterior layer of the CBL, ventral layers II and III of
the CBU and, sparsely, a middle layer of the noduli (Figure 11(c–e)).
The PB is free of TH-immunoreactive fibers.

3.10 | TH immunostaining in the CX of flies
(Diptera)

TH immunostaining was carried out in two dipteran species, the blow-
fly C. erythrocephala and the vinegar fly D. melanogaster (Figure 12). In
both species, innervation of the CX by dopamine-containing neurons has been studied before using antisera against dopamine and TH (Nässel & Elekes, 1992) and, in D. melanogaster, by a TH Gal4 driver line (Mao & Davis, 2009; Omoto et al., 2018). The results of those earlier studies are confirmed here. In both species, all neuropils of the CX are innervated by TH-immunoreactive processes originating from three cell types, PPM3 neurons (equivalent to DP2 neurons in other species) with cell bodies near the lateral tips of the PB (Figure 12(a)), PPL1 neurons (equivalent to DC neurons) with cell bodies anterior-laterally from the calyces (Figure 12(b,f)), and by a single bilateral pair of tritocerebral neurons, termed T1 (Mao & Davis, 2009), not found in other insects studied. Eight PPM3 cells per hemisphere were labeled in both species. Their cell body fibers project anteriorly to the lateral edges of the central body (Figure 12(a)). Processes are sent to the superior medial protocerebrum (Figure 12(c)) and to the lateral accessory lobes (Figure 12(a,c)). Lateral from the EB, the common fascicle of fibers from the neurons separates into two bundles. One bundle, consisting of two fibers in the blowfly, crosses the brain midline dorsally around the EB (Figure 12(a,b)). It encircles and innervates the EB from its outer rim, and sends off side branches to the bulbs lateral from the EB (Figure 12(c,g)). The second bundle proceeds across the midline ventrally between the EB and FB (Figure 12(a,d)). Its fibers give rise to ramifications in ventral layers of the FB, termed FB-III in C. erythrocephala and layers 2 and 4 in Drosophila, following the layering of Wolff et al. (2015). In Drosophila, the four layers of the noduli (NO1, NO2D, NO2V, NO3) as defined by Wolff et al. (2015) are differently supplied by TH-immunolabeled processes.

From a total of 12 PPL1/DC neurons in the blowfly and 9–12 neurons in Drosophila, a small number (two neurons in the blowfly) innervates dorsal layers of the FB (Figure 12(d,h)), while the other PPL1 neurons invade parts of the mushroom bodies (Mao & Davis, 2009). Cell body fibers of PPL1 neurons project medially, give rise to ramifications in the superior medial and intermediate protocerebrum and continue toward the anterior dorsal face of the...
The neurons sparsely innervate dorsal layers of the FB, termed FB-I in the blowfly and layers 5–7 in Drosophila. (Figure 12(d,e,h)).

Finally, a bilateral pair of neurons, termed T1, shows TH immunostaining (Figure 12(b,c,f)). The neurons have cell bodies near the anterior esophageal foramen (Figure 12(c)) and dendritic ramifications in the lateral accessory lobe and the posterior slope of the brain (not shown). Ascending fibers from these neurons project along the brain midline toward the PB. The fibers bifurcate, and each arbor invades one hemisphere of the PB with beaded terminals (Figure 12(b,f)).

**DISCUSSION**

Across dicondylian insects, ranging from firebrats to flies, highly similar types of neurons were immunolabeled with antisera against TH, the rate limiting enzyme of dopamine biosynthesis. Tangential neurons, termed DP2 (PPM3 in flies), with cell bodies near the lateral tips of the CBU, ramifications in the lateral accessory lobes, and axonal projections to ventral layers of the CBU/FB were found in all species, while a second type of neuron, termed DC (PPL1 in flies), with cell
bodies near the calyces, ramifications in the superior protocerebrum, and axonal projections in dorsal layers of the CBU/FB was found in all pterygote species, but not in the firebrat. Based on these two cell types, three layers could be distinguished in the CBU in most species, a dorsal layer, innervated by DC/PPL1 neurons, an intermediate, often less densely labeled layer, and a ventral layer, innervated by TH-immunostained DP2/PPM3 neurons. Although visual input to the CX is prominent in many species (flies, locust, butterfly, cricket), no
obvious differences in TH immunostaining were observed between diurnal (e.g., honeybee) and nocturnal species (e.g., cockroaches), and even in the blind termite TH immunostaining was similar to that in other Dictyoptera. Both types of neuron, however, showed taxon-specific differences in arborization areas outside the CX and, in the case of DP2 neurons, also differences in axonal targets in the central body. Additional types of neuron were only found in two taxa, a system of columnar neurons in the water strider G. lacustris and a tritocerebral neuron innervating the PB in the dipteran species studied. It is, therefore, likely, that DC/PPM1- and DP2/PPM3 neurons play similar roles across insects in arousal and sleep homeostasis, as demonstrated in Drosophila (Liang et al., 2019; Pimentel et al., 2016; Ueno et al., 2012).

4.1 Specificity of immunostaining

TH converts tyrosine into L-DOPA, which is further converted into dopamine by an aromatic acid decarboxylase (Roeder, 2002). Comparison of immunostaining patterns with unrelated antisera against TH and dopamine showed identical patterns of immunolabeled neurons in various insect species (flies: Nässel & Elekes, 1992; cockroach: Hamanaka et al., 2016; locust: Wendt & Homberg, 1992, this study; honeybee: Schäfer & Rehder, 1989; Tedjakumala et al., 2017), indicating that TH labeling is a faithful indicator of neurons containing dopamine and likely releasing dopamine as transmitter. Western blot analysis in Drosophila (Neckameyer & Quinn, 1989), a cricket and a cockroach (Hamanaka et al., 2016), the backswimmer and water strider (this study), revealed single bands with similar molecular size recognized by different TH antibodies indicating lack of crossreactivity with other proteins.

4.2 TH-immunolabeled DP2/PPM3 neurons of the CX

TH-immunoreactive neurons highly similar to DP2 neurons of the locust and PPM3 neurons in flies were found in all insects studied. Common characters of these neurons are the location of their cell bodies near the lateral tips of the PB, anterior/ventrally projecting cell body fibers directed toward the lateral accessory lobe, ramifications in the lateral accessory lobe, and fan-like axonal innervation of ventral layers of the CBU/FB (Figure 13(a)). Except for the firebrat that likely lacks noduli (Pfeiffer & Homberg, 2014), particular layers of the noduli were consistently innervated by at least some DP2/PPM3 neurons. Taxon and partly species-specific differences were found with respect to (a) the number of DP2/PPM3 neurons, (b) additional dendiritic targets besides the lateral accessory lobe, and (c) in many species, axonal innervation of the CBL/EB instead of the CBU/FB by certain members of DP2/PPM3 neurons. While in T. domestica, six to eight DP2 neurons per hemisphere were found, the numbers were much higher in Polynoeoptera, ranging from 25 (stick insect) to over 50 neurons per hemisphere in the desert locust and American cockroach (Hamanaka et al., 2016), but were more moderate again in Holometabola (15 in the honeybee, 8 in flies). This suggests that fine-tuning of CX signaling through neural activity of these neurons has increased from Zygentoma to Polyneoptera, but has decreased again secondarily in Holometabola. Although the innervation areas of DP2/PPM3 neurons outside the CX could not be fully determined owing to overlap with arborizations of other TH-immunolabeled neurons, differences in arborization areas exist between species and between individual neurons within a given species. Most prominent were additional projections of the neurons toward the superior medial protocerebrum in the cricket (Figure 4(a)), the praying mantis (Figure 5(a)), the tobacco moth (Figure 11(c,d)), and the two fly species (Figure 12). Three pairs of PPM3 neurons in Drosophila appear to be the FB4M, FB2A, and FB4L neurons of the Drosophila connectome (Hulse et al., 2020; Scheffer et al., 2020). All three types have prominent processes in the superior medial and intermediate protocerebrum, the lateral accessory lobe, and the wedge, FB4L neurons, in addition, in the crepine (Scheffer et al., 2020).

In the whirligig beetle, dendritic projections of apparently all DP2 neurons extended to an area in close proximity to the mushroom body calyces (Figure 10(g)). Similar projections were not found in other beetles or any other species studied. These projections likely correspond to the visual supply of the mushroom bodies in these insects (Lin &...
Tyrosine hydroxylase (TH) immunostaining in the central complex of the sphinx moth, *Manduca sexta*. (a) Partial reconstruction of TH-immunoreactive DC-type neurons with somata lateral to the calyces (CA), posterior view. Cell body fibers project dorsomedially and give rise to ramifications in the superior medial protocerebrum (SMP). The main fibers cross the brain midline in a commissure dorsally from the central body and give off side branches into the upper division of the central body (CBU). (b) Stack of 21 optical sections illustrating the innervation of layer I of the CBU by side branches from commissural fibers of DC neurons. (c–e) Confocal images from horizontal sections through the central body. TH immunostaining is shown in magenta, phalloidin fluorescence in green. (c) Stack of 15 optical sections through the central body showing TH immunostaining in the lower division of the central body (CBL) and in layer I of the CBU. Staining in the CBL originates from DP2 neurons. Projections from these neurons also extend to the lateral accessory lobes (asterisks, LAL) and to the superior clamp lateral and dorsal from the central body (arrowheads). (d) Stack of 15 optical sections at a more ventral plane, illustrating immunostaining in layers I-III of the CBU (I, II, III). In this plane, the fascicle of cell body fibers of DP2 neurons entering the LAL (yellow arrows) is visible. (e) Single optical section at a further ventral plane, showing sparse immunostaining in a middle layer of the noduli (NO). d, dorsal; ML, medial lobe; p, posterior. Scale bars = 100 μm (a–d), 30 μm (e) [Color figure can be viewed at wileyonlinelibrary.com]
Strausfeld, 2012), which is fundamentally different from the olfactory dominated mushroom bodies in other insects. Because input to these calyces originates exclusively from the dorsal eyes above the water surface but not from the ventral eyes, directed toward objects below the water surface, Lin and Strausfeld (2012) proposed a role of their mushroom bodies in certain aspects of visual spatial orientation. Given the prominent role of the CX in spatial navigation, enhanced connections between mushroom bodies and CX in whirligig beetles might reflect a visual navigation network encompassing both brain areas.

**FIGURE 12** Legend on next page.
In the cricket, locust, cockroach, water strider, backswimmer, honeybee, certain beetles, the sphinx moth, and the two fly species one or two DP2/PPM3 neurons innervate the CBL/EB instead of the CBU/FB. This corresponds with different dendritic targets of these neurons outside the CX. In the locust, a pair of DP2 neurons, termed TL5, innervate the CBL. These neurons have dendritic ramifications in the ipsilateral hemisphere of the PB (Müller et al., 1997). Similar neurons might be present in damselflies (Figure 3). In Drosophila, PPM3 neurons projecting to the EB were morphologically identified as two pairs of ExR2 neurons, also termed PPM3-EB (Omoto et al., 2018). These neurons innervate the bulbs, the crepine, the superior medial protocerebrum, and the lateral accessory lobes (Omoto et al., 2018). Whether innervation of the CBL/EB has occurred once in insect evolution and was then lost secondarily in certain species or has evolved independently several times, remains to be shown.

The functions of DP2/PPM3 neurons have only been addressed in Drosophila and to some extend in the desert locust. In the locust, DP2 neurons are probably not directly involved in sky compass signaling, because they were never encountered among intracellularly recorded CX neurons responding to sky compass stimuli (Heinze & Homberg, 2009; Pegel et al., 2018; Vitzthum et al., 2002). TL5 neurons, innervating the CBL, show regular firing activity, no response to lights on/off and no preference for polarization angle, suggesting that they are not part of the sky compass network of the CX but rather provide modulatory input to the CBL (Vitzthum et al., 2002). In Drosophila PPM3-EB, neurons activate ring neurons of the EB and thereby promote locomotor activity in response to ethanol (Kong et al., 2010). Liang et al. (2019) showed that PPM3-EB neurons are under circadian control and mediate the morning and evening peaks of daily locomotor activity through activation of EB ring neurons.

4.3 | TH-immunolabeled DC/PPL1 neurons of the CX

In contrast to the DP2/PPM3 neurons, only a fraction of DC/PPL1 neurons targets the central body. Most of these neurons bypass the CX, but innervate various parts of the mushroom body with a prominent role in olfactory memory formation (Huetteroth et al., 2015; Siju et al., 2021). These neurons are already present in the flightless firebrat (Figure 2(d,e)), but here, none of the DC neurons innervate the central body. In all pterygote species, however, one to five bilateral pairs of DC/PPL1 neurons innervate dorsal layers of the CBU/FB. Consistent features of these neurons are cell bodies below the calyx of the mushroom body, dendritic ramifications in the superior medial and/or intermediate protocerebrum, and axonal projections along the dorsal/anterior face of the CBU with side branches innervating dorsal layers of the CBU (Figure 13(b)). There is little variation in the number of these neurons, only one bilateral pair is TH-immunoreactive in the beetle T. molitor, whereas two subtypes, DC1 and DC2 can be distinguished in the locust based on slightly different axonal paths (Wendt & Homberg, 1992). In the blowfly, two pairs of PPL1 neurons innervate the FB (Figure 12). The functional role of these neurons has only been addressed in Drosophila. Here, a bilateral pair of dopaminergic PPL1 neurons promotes wakefulness and arousal by inhibiting sleep-promoting neurons in the dorsal FB (Liu, Liu, et al., 2012; Ueno et al., 2012). The neurons are apparently not under circadian control but are part of a neural network regulating sleep homeostasis (Donlea, 2017; Dubowy & Sehgal, 2017).

4.4 | Columnar neurons in the water strider

In addition to DC- and DP2-type neurons, the central body of the water strider exhibits TH immunostaining in a set of columnar neurons connecting the PB, the CBU and the noduli (Figure 13(c)). The neurons are...
likely homologous to CPU4- or CPU5 neurons of locusts (Heinze & Homberg, 2008) and P-FN neurons in flies (Wolff & Rubin, 2018). CPU4 neurons of the locust exhibit serotonin- and Dip-allatostatin immuno-staining (Homberg, 1991; Vitzthum et al., 1996). This suggests that neurons homologous to the TH-immunolabeled columnar neurons of the water strider are present in other insect species but likely use a different neurotransmitter. In locusts, CPU4 neurons are part of the sky-compass network of the CX (Heinze & Homberg, 2009). In a computational model, they have been proposed to serve a memory function during path integration (Stone et al., 2017).

4.5 Immunolabeled T1 neurons in flies

Only some species showed immunolabeling for TH in the PB. In S. gregaria, A. imperator, and C. viridis, these innervations originate from...
side branches of DP2 neurons in the posterior pars intercerebralis. In the water strider G. lacustris, strong immunoreactivity in the PB results from the immunolabeled columnar neurons. In the two dipterans studied, a novel type of TH immunostained tritocerebral neuron with only one neuron per brain hemisphere (Mao & Davis, 2009; Wolff et al., 2015) was stained (Figure 13d)). As shown here and by Wolff et al. (2015), the neurons have ramifications in the lateral accessory lobe and posterior slope and send presynaptic endings into all slices of the PB. Similar neurons have not been found in other insects so far; therefore, T1 may be dipteran-specific types of neuron. The functional role of T1 in Drosophila has been addressed by Alekseyenko et al. (2013) and, more recently, by Duhart et al. (2020). Selective activation and inactivation of the T1 neurons increased aggressiveness in male flies (Alekseyenko et al., 2013), and activation furthermore, reduced sleep in male but not in female flies (Duhart et al., 2020).

5 | CONCLUSIONS

Comparison of TH immunostaining in the CX of dicondylian insects suggests that dopaminergic neurons innervating the CX are highly conserved across insects, similar to recently reported GABA-immunoreactive neurons (Homberg et al., 2018). Innervation by one type of these neurons, termed DC/PPL1, was absent in firebrats and likely evolved during the transition to flight. Further evolutionary changes occurred regarding the number of immunolabeled neurons and, as shown before for serotonin-containing neurons of the antennal lobe (Dacks et al., 2006), by changes in the arborization patterns of the neurons both at their input and output sites. Dopaminergic neurons of the CX are apparently not integral elements of the head-direction network within the CX, but seem to modulate neural activity levels state dependently. As shown in Drosophila DP2/PPM3, neurons are part of efferent outputs of the circadian clock, while DC/PPL1 neurons are part of a neural network regulating sleep homeostasis. Both cell types raise activity levels in CX neurons, which in turn leads to arousal, promotes wakefulness, locomotor activity, and other motor actions. Based on similar effects resulting from manipulations of dopamine neurotransmission in the brains of other insect species (Mustard et al., 2012; Verlinden, 2018), it is likely that similar roles apply to dopaminergic neurons of the CX across all insect species.

ACKNOWLEDGMENTS

The authors are grateful to Drs Christian Bökel, Andreas Brune, and Monika Stengl for providing termites, flies, and sphinx moths. Funding was obtained from Deutsche Forschungsgemeinschaft, grant number: HO 950/24-1 and HO 950/26-1. Open access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Uwe Homberg: Study concept and design. Josephine Timm, Mara Scherner, Jannik Matschke, and Martina Kern: Acquisition of data. Josephine Timm, Mara Scherner, Jannik Matschke, and Uwe Homberg: Data analysis and interpretation. Josephine Timm and Uwe Homberg: Drafting the manuscript. Uwe Homberg and Josephine Timm: Review and editing.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/cne.25151.

DATA AVAILABILITY STATEMENT

All relevant data are available from the corresponding author.

ORCID

Uwe Homberg https://orcid.org/0000-0002-8229-7236

REFERENCES

Alekseyenko, O. V., Chan, Y.-C., Li, R., & Kravitz, A. (2013). Single dopaminergic neurons that modulate aggression in Drosophila. Proceedings of the National Academy of Sciences of the United States of America, 110, 6151–6156.

Aso, Y., & Rubin, G. (2016). Dopaminergic neurons write and update memories with cell-type-specific rules. eLife, 5, e16135.

Boto, T., Stahl, A., Zhang, X., Louis, T., & Tomchik, S. M. (2019). Independent contributions of discrete dopaminergic circuits to cellular plasticity, memory strength, and valence in Drosophila. Cell Reports, 27, 2014–2021.

Calvo, A. C., Pey, A. L., Miranda-Vizuete, A., Daskeland, A. P., & Martinez. A. (2011). Divergence in enzyme regulation between Caenorhabditis elegans and human tyrosine hydroxylase, the key enzyme in the synthesis of dopamine. Biochemical Journal, 434, 133–141.

Claassen, D. E., & Kammer, A. E. (1986). Effects of octopamine, dopamine, and serotonin on production of flight motor output by thoracic ganglia of Manduca sexta. Journal of Neurobiology, 17, 1–14.

Dacks, A. M., Christensen, T. A., & Hildebrand, J. G. (2006). Phylogeny of a serotonin-immunoreactive neuron in the primary olfactory center of the insect brain. Journal of Comparative Neurology, 498, 727–746.

Das, G., Lin, S., & Waddell, S. (2016). Remembering components of food in Drosophila. Frontiers in Integrative Neuroscience, 10, 4.

Donlea, J. M. (2017). Neuronal and molecular mechanisms of sleep homeostasis. Current Opinion in Insect Science, 24, 51–57.

Dubowy, C., & Sehgal, A. (2017). Circadian rhythms and sleep in Drosophila melanogaster. Genetics, 205, 1373–1397.

Duhart, J. M., Baccini, V., Zhang, Y., Machado, D. R., & Koh, K. (2020). Modulation of sleep-courtship balance by nutritional status in Drosophila. eLife, 9, e60853.

Frisch, C., & Dolan, R. J. (1998). Images of psychopharmacology. Current Opinion in Neurobiology, 8, 259–262.

Green, J., Vijayan, V., Pires, P. M., Adachi, A., & Maimon, G. (2019). A neural heading estimate is compared with an internal goal to guide oriented navigation. Nature Neuroscience, 22, 1460–1468.

Hamanaka, Y., Minoura, R., Nishino, H., Miura, T., & Mizunami, M. (2016). Dopamine- and tyrosine hydroxylase-immunoreactive neurons in the brain of the American cockroach, Periplaneta americana. PLoS One, 11, e0160531.

Hanesch, U., Fischbach, K.-F., & Heisenberg, M. (1989). Neuronal architecture of the central complex in Drosophila melanogaster. Cell and Tissue Research, 257, 343–366.

Harano, K., Sasaki, M., Nagao, T., & Sasaki, K. (2008). Dopamine influences locomotor activity in honeybee queen: Implications for a behavioural change after mating. Physiological Entomology, 33, 395–399.
Hartenstein, V., Cruz, L., Lovick, J. K., & Guo, M. (2017). Developmental analysis of the dopamine-containing neurons of the Drosophila brain. Journal of Comparative Neurology, 525, 363–379.

Heinez, S., & Homberg, U. (2007). Maplike representation of cephalic E-vector orientations in the brain of an insect. Science, 315, 995–997.

Heinez, S., & Homberg, U. (2008). Neuroarchitecture of the central complex of the desert locust: Intrinsic and columnar neurons. Journal of Comparative Neurology, 511, 454–478.

Heinez, S., & Homberg, U. (2009). Linking the input to the output: New sets of neurons complement the polarization vision network in the locust central complex. Journal of Neuroscience, 29, 4911–4921.

Helfrich-Förster, C. (2018). Sleep in insects. Annual Review of Entomology, 63, 69–86.

Homberg, U. (1991). Neuroarchitecture of the central complex in the brain of the locust Schistocerca gregaria and S. americana as revealed by serotonin immunocytochemistry. Journal of Comparative Neurology, 303, 245–254.

Homberg, U., Humberg, T.-H., Seyfarth, J., Bode, K., & Quintero-Pérez, M. (1997). Neuroarchitecture of the central complex in the brain of the desert locust. Journal of Comparative Neurology, 384, 287–303.

Huetteroth, W., Perisse, E., Lin, S., Klappenbach, M., Burke, C., & Waddell, S. (2015). Cephalic E-vector orientation in Schistocerca gregaria and Schistocerca americana: A regulator of arousal in the fruit fly. Journal of Neuroscience, 35, 7377–7384.

Lawrence, A.-M., & Besir, H. (2009). Staining of proteins in gels with Coomassie G-250 without organic solvent and acetic acid. Journal of Visualized Experiments, 30, e1350.

Lin, C., & Strausfeld, N. J. (2012). Visual inputs to the mushroom body calyces of the whirlygig beetle Dineutus sublineatus: Modality switching in an insect. Journal of Comparative Neurology, 520, 2562–2574.

Liu, C., Plaçaís, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., Siwanowicz, I., Rubin, G. M., Prent, T., & Tanimoto, H. (2012). A subset of dopamine neurons signals reward for odour memory in Drosophila. Nature, 488, 512–516.

Liu, Q., Liu, S., Kodama, L., Driscoll, M. R., & Wu, M. N. (2012). Two dopaminergic neurons signal to the dorsal fan-shaped body to promote wakefulness in Drosophila. Current Biology, 22, 2114–2123.

Mao, Z., & Davis, R. L. (2009). Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: Anatomical and physiological heterogeneity. Frontiers in Neural Circuits, 3, 1–17.

Martin, J. P., Guo, P., Mu, L., Harley, C. M., & Ritzmann, R. E. (2015). Central-complex control of movement in the freely walking cockroach. Current Biology, 25, 2795–2803.

Müller, M., Homberg, U., & Kühn, A. (1997). Neuroarchitecture of the lower division of the central body in the brain of the locust Schistocerca gregaria. Cell and Tissue Research, 288, 159–176.

Mustard, J. A., Verzgoz, V., Mesce, K. A., Klukas, K. A., Beggs, K. T., Geddes, L. H., McQuillan, H. J., & Mercer, A. R. (2012). Dopamine signalling in the bee. In C. G. Galizia, D. Eisenhardt, & M. Giurfa (Eds.), Honeybee neurobiology and behavior (pp. 199–209). Springer.

Nässel, D. R., & Elekes, K. (1992). Aminergic neurons in the brain of blowflies and Drosophila: Dopaminergic and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. Cell and Tissue Research, 267, 147–167.

Neckameyer, W. S., & Quinn, W. G. (1989). Isolation and characterization of the gene for Drosophila tyrosine hydroxylase. Neuron, 2, 1167–1175.

Oamoto, J. J., Nguyen, B.-C. M., Kandimalla, P., Lovick, J. K., Donlea, J. M., & Hartenstein, V. (2018). Neuronal constituents and putative interactions within the Drosophila ellipsoid body neuropil. Frontiers in Neural Circuits, 12, 1–26.

Otto, N., Pleijzier, M. W., Morgan, I. C., Edmonton-Stait, A. J., Heinz, K. J., Stark, I., Dempsey, G., Masayoshi, I., Kapoor, I., Hsu, J., Schlegel, P. M., Bates, A. S., Feng, L., Costa, M., Ito, K., Bock, D. D., Rubin, G. M., Jeffries, S. X. E., & Waddell, S. (2020). Input connectivity reveals additional heterogeneity of dopaminergic reinforcement in Drosophila. Current Biology, 30, 3200–3211.

Pegel, U., Pfeiffer, K., & Homberg, U. (2018). Integration of cephalic compass cues in the central complex of the locust brain. Journal of Experimental Biology, 221, 1–15.

Pendleton, R. G., Rasheed, A., Sardina, T., Tully, T., & Hillman, R. (2002). Effects of tyrosine hydroxylase mutants on locomotor activity in Drosophila: A study in functional genomics. Behavior Genetics, 32, 89–94.

Pfeiffer, K., & Homberg, U. (2014). Organization and functional roles of the central complex in the insect brain. Annual Review of Entomology, 59, 165–184.

Pimentel, D., Donlea, J. M., Talbot, C. B., Song, S. M., Thurston, A. J. F., & Miesenböck, G. (2016). Operation of a homeostatic sleep switch. Nature, 536, 333–337.

Pisokas, I., Heinez, S., & Webb, B. (2020). The head direction circuit of two insect species. eLife, 9, e53985.

Potdar, S., & Sheeba, V. (2018). Wakefulness is promoted during the day by PDFR signalling to dopaminergic neurons in Drosophila melanogaster. eNeuro, 5(e0129-18), 2018.

Rangel-Barajas, C., Coronel, I., & Florán, B. (2015). Dopamine receptors and neurodegeneration. Aging Disorders, 6, 349–368.

Riemensperger, T., Isabel, G., Coulon, H., Neuser, K., Seugnet, L., Kume, K., Iche-Torres, M., Cassar, M., Strauss, R., Prent, T., Hirsh, J., & Birman, S. (2011). Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. Proceedings of the National Academy of Sciences of the United States of America, 108, 834–839.

Roeder, T. (2002). Biochemistry and molecular biology of receptors for biogenic amines in locusts. Microscopy Research and Technique, 56, 237–247.

Ryczko, D., Cone, J. J., Alpert, M. H., Goetz, L., Auclair, F., Dubé, C., Parent, M., Roitman, M. F., Alford, S., & Dubuc, R. (2016). A descending dopamine pathway conserved from basal vertebrates to mammals. Proceedings of the National Academy of Sciences of the United States of America, 113, E2440–E2449.

Schäfer, S., & Rehder, V. (1989). Dopamine-like immunoreactivity in the brain and subesophageal ganglion of the honeybee. Journal of Comparative Neurology, 280, 43–59.

Scheffer, L. K., Xu, C. S., Januszewski, M., Lu, Z., Takemura, S., Hayworth, K. J., Huang, G. B., Shinomiya, K., Maitîn-Shepard, J., Berg, S., Clements, J., Hubbard, P. M., Katz, W. T., Umaylam, L., Zhao, T., Ackerman, D., Blakey, T., Bogovíc, J., Dolafi, T., & Plaza, S. M. (2020). A connectome of the adult Drosophila central brain. eLife, 9, e57443.

Schürmann, F. W., Elekes, K., & Geffard, M. (1989). Dopamine-like immunoreactivity in the bee brain. Cell and Tissue Research, 256, 399–410.
Seelig, J. D., & Jayaraman, V. (2015). Neural dynamics for landmark orientation and angular path integration. Nature, 521, 186–191.

Shiozaki, H. M., Ohta, K., & Kazama, H. (2020). A multi-regional network encoding heading and steering maneuvers in Drosophila. Neuron, 106, 1–16.

Siju, K. P., de Backer, J.-F., & Grunwald Kadow, I. C. (2021). Dopamine modulation of sensory processing and adaptive behavior in flies. Cell and Tissue Research, 383, 207–225.

Smeets, W. J. A. J., & González, A. (2000). Catecholamine systems in the brain of vertebrates: New perspectives through a comparative approach. Brain Research Reviews, 33, 308–379.

Spanagel, R., & Weiss, F. (1999). The dopamine hypothesis of reward: Past and current status. Trends in Neurosciences, 22, 521–527.

Stone, T., Webb, B., Adden, A., Wedding, N. B., Honkanen, A., Templin, R., Wcislo, W., Sicmeca, L., Warrant, E., & Heinze, S. (2017). An anatomically constrained model for path integration in the bee brain. Current Biology, 27, 3069–3085.

Sullivan, L. F., Warren, T. L., & Doe, C. Q. (2019). Temporal identity establishes columnar neuron morphology, connectivity, and function in a Drosophila navigation circuit. eLife, 8, e43482.

Tedjakumala, S. R., Rouquette, J., Boizeau, M.-L., Mesce, K. A., Hotier, L., Massou, I., & Giurfa, M. (2017). A tyrosine-hydroxylase characterization of dopaminergic neurons in the honey bee brain. Frontiers in Systems Neuroscience, 11, 1–17.

Triphan, T., Poeck, B., Neuser, K., & Strauss, R. (2010). Visual targeting of motor actions in climbing Drosophila. Current Biology, 20, 663–668.

Ueno, T., Tomita, J., Tanimoto, H., Endo, K., Ito, K., Kume, S., & Kume, K. (2012). Identification of a dopamine pathway that regulates sleep and arousal in Drosophila. Nature Neuroscience, 15, 1516–1523.

Utz, S., Huetteroth, W., Vömel, M., & Schachtner, J. (2008). Masallatotropin in the developing antennal lobe of the sphinx moth Manduca sexta: Distribution, time course, developmental regulation, and colocalization with other neuropeptides. Developmental Neurobiology, 68, 123–142.

van Swinderen, B., & Andretic, R. (2011). Dopamine in Drosophila: Setting arousal thresholds in a miniature brain. Proceedings of the Royal Society B, 278, 906–913.

Varga, A. G., & Ritzmann, R. E. (2016). Cellular basis of head direction and contextual cues in the insect brain. Current Biology, 26, 1816–1828.

Verlinden, H. (2018). Dopamine signaling in locusts and other insects. Insect Biochemistry and Molecular Biology, 97, 40–52.

Vitzthum, H., Homberg, U., & Agricola, H. (1996). Distribution of Dipallatostatin I-like immunoreactivity in the brain of the locust Schistocerca gregaria with detailed analysis of immunostaining in the central complex. Journal of Comparative Neurology, 369, 419–437.

Vitzthum, H., Müller, M., & Homberg, U. (2002). Neurons of the central complex of the locust Schistocerca gregaria are sensitive to polarized light. Journal of Neuroscience, 22, 1114–1125.

Waddell, S. (2013). Reinforcement signaling in Drosophila; dopamine does it all after all. Current Opinion in Neurobiology, 23, 324–329.

Wendt, B., & Homberg, U. (1992). Immunocytochemistry of dopamine in the brain of the locust Schistocerca gregaria. Journal of Comparative Neurology, 321, 387–403.

Wolff, T., Iyer, N. A., & Rubin, G. M. (2015). Neuroarchitecture and neuroanatomy of the Drosophila central complex: A GAL4-based dissection of protocerebral bridge neurons and circuits. Journal of Comparative Neurology, 523, 997–1037.

Wolff, T., & Rubin, G. M. (2018). Neuroarchitecture of the Drosophila central complex: A catalog of nodulus and asymmetrical body neurons and a revision of the protocerebral bridge catalog. Journal of Comparative Neurology, 526, 2585–2611.

Wright, G. (2011). The role of dopamine and serotonin in conditioned food aversion learning in the honeybee. Communicative and Integrative Biology, 4, 318–320.

Yamamoto, K., & Vernier, P. (2011). The evolution of dopamine systems in chordates. Frontiers in Neuroanatomy, 5, 21.

Yamamoto, S., & Seto, E. S. (2014). Dopamine dynamics and signaling in Drosophila: An overview of genes, drugs and behavioral paradigms. Experimental Animals, 63, 107–119.

Zitrell, F., Pfeiffer, K., & Homberg, U. (2020). Matched-filter coding of sky polarization results in an internal sun compass in the brain of the desert locust. Proceedings of the National Academy of Sciences of the United States of America, 117, 25810–25817.

How to cite this article: Timm J, Scherner M, Matschke J, Kern M, Homberg U. Tyrosine hydroxylase immunostaining in the central complex of dicondyliana insects. J Comp Neurol. 2021;529:3131–3154. https://doi.org/10.1002/cne.25151