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REGULAR ARTICLE

Tyrosine hydroxylase-immunoreactive neurons in the mushroom body of the field cricket, *Gryllus bimaculatus*

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Abstract

The mushroom body of the insect brain participates in processing and integrating multimodal sensory information and in various forms of learning. In the field cricket, *Gryllus bimaculatus*, dopamine plays a crucial role in aversive memory formation. However, the morphologies of dopamine neurons projecting to the mushroom body and their potential target neurons, the Kenyon cells, have not been characterized. Golgi impregnations revealed two classes of Kenyon cells (types I and II) and five different types of extrinsic fibers in the mushroom body. Type I cells, which are further divided into two subtypes (types I core and I surface), extend their dendrites into the anterior calyx, whereas type II cells extend many bushy dendritic branches into the posterior calyx. Axons of the two classes bifurcate between the pedunculus and lobes to form the vertical, medial and γ lobes. Immunocytochemistry to tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine biosynthesis, revealed the following four distinct classes of neurons: (1) TH-SLP projecting to the distal vertical lobe; (2) TH-IP1 extending to the medial and γ lobes; (3) TH-IP2 projecting to the basal vertical lobe; and (4) a multiglomerular projection neuron invading the anterior calyx and the lateral horn (TH-MPN). We previously proposed a model in the field cricket in which the efficiency of synapses from Kenyon cells transmitting a relevant sensory stimulus to output neurons commanding an appropriate behavioral reaction can be modified by dopaminergic neurons mediating aversive signals and here, we provide putative neural substrates for the cricket's aversive learning. These will be instrumental in understanding the principle of aversive memory formation in this model species.

Keywords Insect · Dopamine · Aversive learning · Prediction error · Kenyon cell

Introduction

Learning, such as Pavlovian (or classical) conditioning, is a ubiquitous phenomenon in animal behavior, in which the association of a certain neutral sensory stimulus (conditioned stimulus, CS) with a biologically relevant stimulus (unconditioned stimulus, US) provides either aversive or appetitive predictive value to the CS. Following such association, the CS alone allows the animal to retrieve an aversive or appetitive memory and choose an appropriate behavior, i.e., a conditioned response (CR). There are, however, two immediate questions, which are as follows: (1) how are the CS and US associated in the brain to achieve conditioning? and (2) how is the experience or memory stored in the brain to optimize the animal's behavior in the future. To address these questions, we need to explore and identify the neural circuit mechanisms underlying conditioning. In mammals, dopamine neurons in the ventral tegmental area of the midbrain are crucial for mediating appetitive and aversive reinforcement (Schultz 2006, 2013; Steinberg et al. 2013; Matsumoto et al. 2016) and thus, much effort has been focused on the roles of these neurons in associative learning. However, the size and complexity of mammalian brains prevent us from identifying sets of neurons participating in learning and memory. As an alternative, insects provide us a favorable opportunity to approach neural circuits, thanks to the relatively small number of neurons constituting their numerically simple but elaborate brain (Mizunami et al. 1999, 2004; Hige 2018). Accordingly, for a long time, insects have been used as a model to unveil the basic principle of associative learning (Menzel et al. 2006; Giurfa 2007; Mizunami and Matsumoto

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2010; Mizunami et al. 2013, 2015; Waddell 2013; Owald and Waddell 2015; Cognigni et al. 2018; Hige 2018).

The field cricket, *Gryllus bimaculatus*, is one of the most intensively studied animals with respect to associative learning because of its excellent capabilities in olfactory and visual learning, as well as second-order conditioning (Unoki et al. 2005, 2006; Mizunami et al. 2009, 2013, 2015; Nakatani et al. 2009; Mizunami and Matsumoto 2010; Terao et al. 2015; Terao and Mizunami 2017). Recent molecular biological studies using both the CRISPR/Cas9 system and RNAi technique validated our previous pharmacological data and concluded that aversive reinforcement is mediated exclusively by dopaminergic neurons via the dopamine receptor, Dop1, in the field cricket (Awata et al. 2015, 2016). Dopaminergic systems mediate aversive reinforcement also in the fruit fly (Schwaerzel et al. 2003; Aso et al. 2012; Vogt et al. 2014), honey bee (Vergoz et al. 2007) and mammals (Schultz 2006, 2013; Matsumoto et al. 2016). Moreover, crickets and mammals appear to share common computational principles in associative learning, for which the applicability of a prediction error theory to both the cricket's appetitive (Terao et al. 2015) and aversive (Terao and Mizunami 2017) conditioning provide evidence. This theory is based on the idea that a discrepancy or an error between the actual US and the predicted US from the CS determines whether learning occurs or not (Rescorla and Wagner 1972; Schultz 2006, 2013). Validation of this theory has been unequivocally demonstrated in the field cricket. Moreover, octopamine and dopamine are suggested to mediate an appetitive and aversive prediction error signal, respectively (Terao et al. 2015; Terao and Mizunami 2017). Thus, this insect has now emerged as one of the most intriguing experimental subjects to study the computational mechanisms of the prediction error underlying associative learning.

The Dop1 dopamine receptor, which mediates aversive learning in the field cricket (Awata et al. 2015, 2016), expresses predominantly in a subset of the intrinsic Kenyon cells constituting the mushroom body (Hamada et al. 2009). As shown in different insect species, this neuropil plays an important role as a center for high-order olfactory and multisensory information processing as well as various forms of learning (Heisenberg 2003; Giurfa 2007; Mizunami et al. 2013, 2015; Waddell 2013; Owald and Waddell 2015; Cognigni et al. 2018; Hige 2018). The mushroom body comprises three distinct regions, namely the calyx, pedunculus and lobe. The Kenyon cells receive sensory inputs in the calyx via projection neurons and form synapses upon the mushroom body's output neurons in the lobes, a part of the latter functioning as a commander of the CR after the conditioning (Mizunami and Matsumoto 2010). We suggested in our model that dopaminergic neurons encoding aversion and/or an aversive prediction error probably innervate the lobes (Terao and Mizunami 2017). The dopaminergic neurons are considered to modulate the efficiency of synapses between the Kenyon cells (representing the CS) and the mushroom body's output neurons (commanding the CR) in the field cricket (Terao and Mizunami 2017). The critical roles of the lobes in the association between the CS and the US have been established in the fruit fly (Owald and Waddell 2015; Cognigni et al. 2018; Hige 2018). However, dopamine neurons in the mushroom body have yet to be characterized in the field cricket.

In the insect's small brain, a small number of neurons or even a singly identifiable neuron often serves as a functional unit responsible for information processing (Mizunami et al. 2004). Thus, insects are ideal experimental subjects to monitor neural plasticity that occurs during the process of memory formation especially at single-cell resolution. In particular, crickets provide an opportunity to study cellular mechanisms by which dopaminergic neurons mediate aversive reinforcement and/or aversive prediction error signals (Mizunami et al. 2015). For this purpose, it is a prerequisite to identify dopaminergic neurons projecting to the mushroom body. In the present account, we studied putative dopaminergic neurons in the brain of the field cricket with special attention to the mushroom body and the antennal lobe, both being involved in olfactory conditioning, using an antiserum against tyrosine hydroxylase, a rate-limiting enzyme in dopamine biosynthesis (Blenau and Baumann 2001; Roeder 2002). Mapping the dopaminergic neurons associated with these neuropil structures will, we predict, provide us with the bases to efficiently probe neural mechanisms underlying aversive reinforcement and the prediction error and, thereby, accelerate an understanding of associative learning.

Materials and methods

Animals

Field crickets (*Gryllus bimaculatus*) were raised in crowded colonies at Hokkaido University (Sapporo, Hokkaido, Japan) under a 12 h:12 h light:dark cycle at 27 ± 1 °C. Adult male crickets less than 7 days after adult eclosion were used for experiments.

Reduced silver impregnation

Cricket brains were carefully dissected out and then fixed in a solution containing 4% paraformaldehyde (PFA), 5% glacial acetic acid and 85% ethanol for 2 days. The tissues were dehydrated and then embedded in paraffin. Reduced silver impregnation was performed on 10- to 16-μm sections as described elsewhere (Mizunami et al. 1998).

Golgi impregnation

To visualize Kenyon cells and extrinsic fibers in the mushroom body, Golgi impregnation technique was employed. The method has been described in detail elsewhere (Mizunami et al. 1998).

Immunoperoxidase staining

Brains were fixed with 4% PFA in 0.067 M phosphate buffer (PB, pH 7.4) overnight at 4 °C. The fixed tissues were embedded in a gelatin/albumin mixture and post-fixed with 7.4% formaldehyde in PB. The post-fixed tissues were sectioned at a thickness of 40–50 μm with a vibrating blade microtome (LinearSlicer PRO 7; Dosaka EM Co., Ltd., Kyoto, Japan). Non-specific binding sites were blocked with 5% normal goat serum (NGS) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.5% Triton X (PBST) for 1 h and then incubated for 2 days at 4 °C with a rabbit polyclonal antiserum against tyrosine hydroxylase (TH; Cat. No. NB300-109, Novus Biologicals, Littleton, CO, USA) in PBST containing 5% NGS, at a working dilution of 1:4000 to 1:5000. After washing in PBST, the sections were incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase (1:200, Jackson ImmunoResearch, West Grove, PA, USA) in PBST containing 5% NGS. After washing in PBST, sections were developed with a solution of 0.032% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 0.1 M Tris-HCl (pH 7.4) containing 0.0145% H_2O_2 and 0.3% nickel ammonium sulfate, followed by washing in PB. The sections were mounted on a gelatin-coated glass slide, dehydrated in a graded ethanol series, cleared in xylene and mounted in Mount-Quick (Daido Sangyo, Tokyo, Japan) beneath cover slips. The specificity of the anti-TH in *G. bimaculatus* brains has been demonstrated by Western blots, which identify a single band of ~ 66 kDa (Hamanaka et al. 2016).

Image capture, drawing and brain nomenclature

DAB-labeled, reduced silver-impregnated or Golgi-impregnated preparations were imaged with a digital camera (DMC-G5, Panasonic, Osaka, Japan) mounted on a compound light microscope (BX60, Olympus, Tokyo, Japan). Several images focused on different planes were manually captured from single sections at intervals of 3–5 μm and then superimposed into a single image using Zerene Stacker software (Zerene Systems LLC, Richland, WA, USA). Neurons were drawn with a camera lucida attached to the compound light microscope. The size, contrast and brightness of the images were adjusted using Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) and Corel Draw X4 (Corel, Ottawa, ON, Canada).

Neuropils and tracts were named in accordance with the insect brain name working group (Ito et al. 2014).

Results

Gross neuroanatomy of the field cricket's mushroom body

The mushroom body is a paired neuropil structure (Fig. 1) serving as a high-order center for multimodal sensory information processing and associative learning. This paired neuropil structure contains the following two types of neurons: (1) intrinsic Kenyon cells and (2) extrinsic neurons, the former determining the shape of the mushroom body and the latter connecting it to the other brain neuropils. As in other insects, the mushroom body of the cricket is divided into three main compartments, i.e., the calyx, pedunculus and lobe. The calyx is further subdivided into the anterior calyx (ACA) and the posterior calyx (PCA) Fig. 1). The former is mainly invaded by a group of projection neurons extending their dendrites in the antennal lobe, while the latter is invaded by another group of projection neurons originating from the lobus glomerulatus (Frambach and Schürmann 2004). Golgi impregnation revealed two distinct classes of Kenyon cells (types I and II) in the field cricket's mushroom body (Fig. 2). These extend dendrites to either the anterior or posterior calyx and uniformly project their axons arranged in parallel toward the pedunculus. The axons bifurcate at a transition zone between the pedunculus and the lobes to form the vertical and medial lobes or the vertical and γ lobes, respectively. Type I Kenyon cells, which are further divided into two subtypes, i.e., type I core (Fig. 2a,a') and type I surface (Fig. 2b,b'), have one to a few short dendritic extensions in the anterior calyx (arrow in Fig. 2a,b,a',b') and their axons run in the pedunculus. Type I surface Kenyon cells extend the dendrites exclusively to the anterior calyx (arrows in Fig. 2b,b'), the number of which is much larger than those of type I core Kenyon cells (Fig. 2a,a'). On the other hand, type II Kenyon cells form bushy ramifications in the posterior calyx (Fig. 2c,c'). Axons of the type I core/surface form sparse ramifications in the distal regions of the vertical and medial lobes (Fig. 2d,d') while those of the type II bear many varicose fibers along their axons in the γ lobe (Fig. 2e,e'). Projection patterns of respective Kenyon cells are summarized in Fig. 3. Golgi impregnation also visualized distinct sets of extrinsic fibers. In the vertical lobe, two types of fibers were impregnated, one type arborizing perpendicularly against the bundle of Kenyon cell axons at the middle portion of the vertical lobe (arrow in Fig. 4a) and the other arborizing at the lateral tip (arrow in Fig. 4b). The middle part of the medial lobe receives a similar pattern of arborizations as in the vertical lobe (arrow in Fig. 4c). In the posterior calyx, a system of blebby fibers

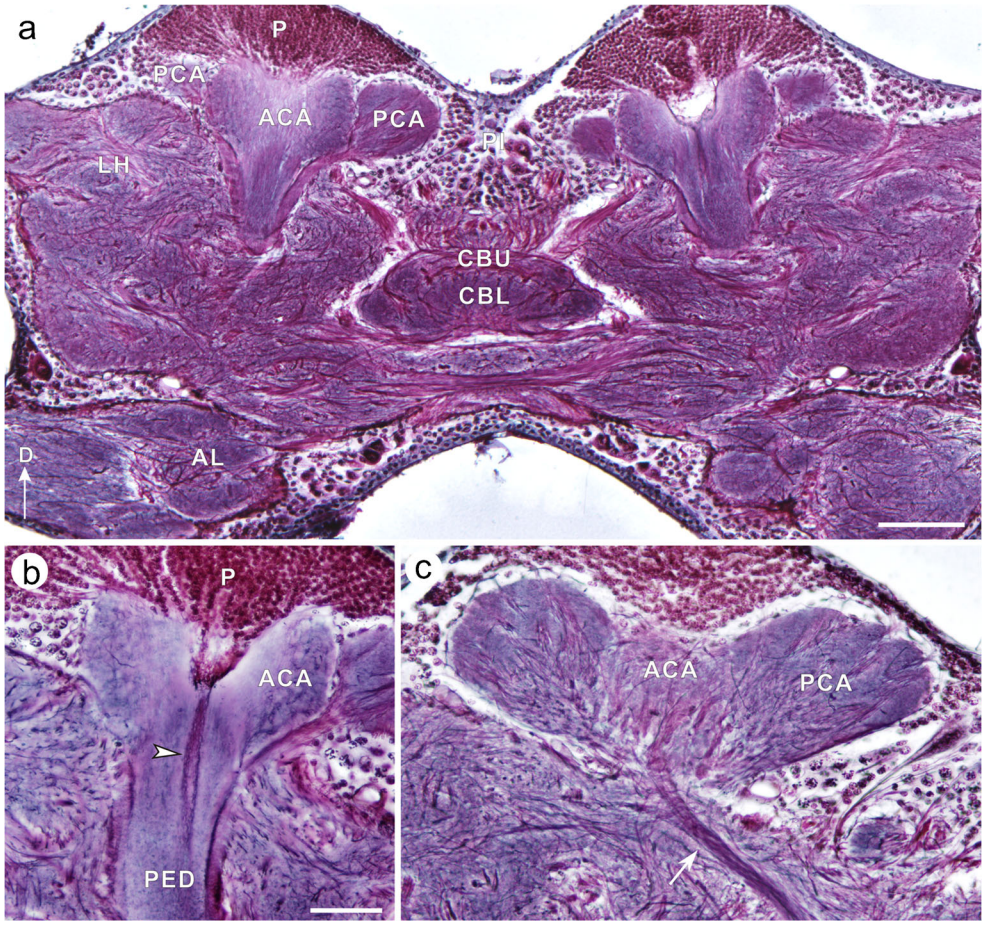


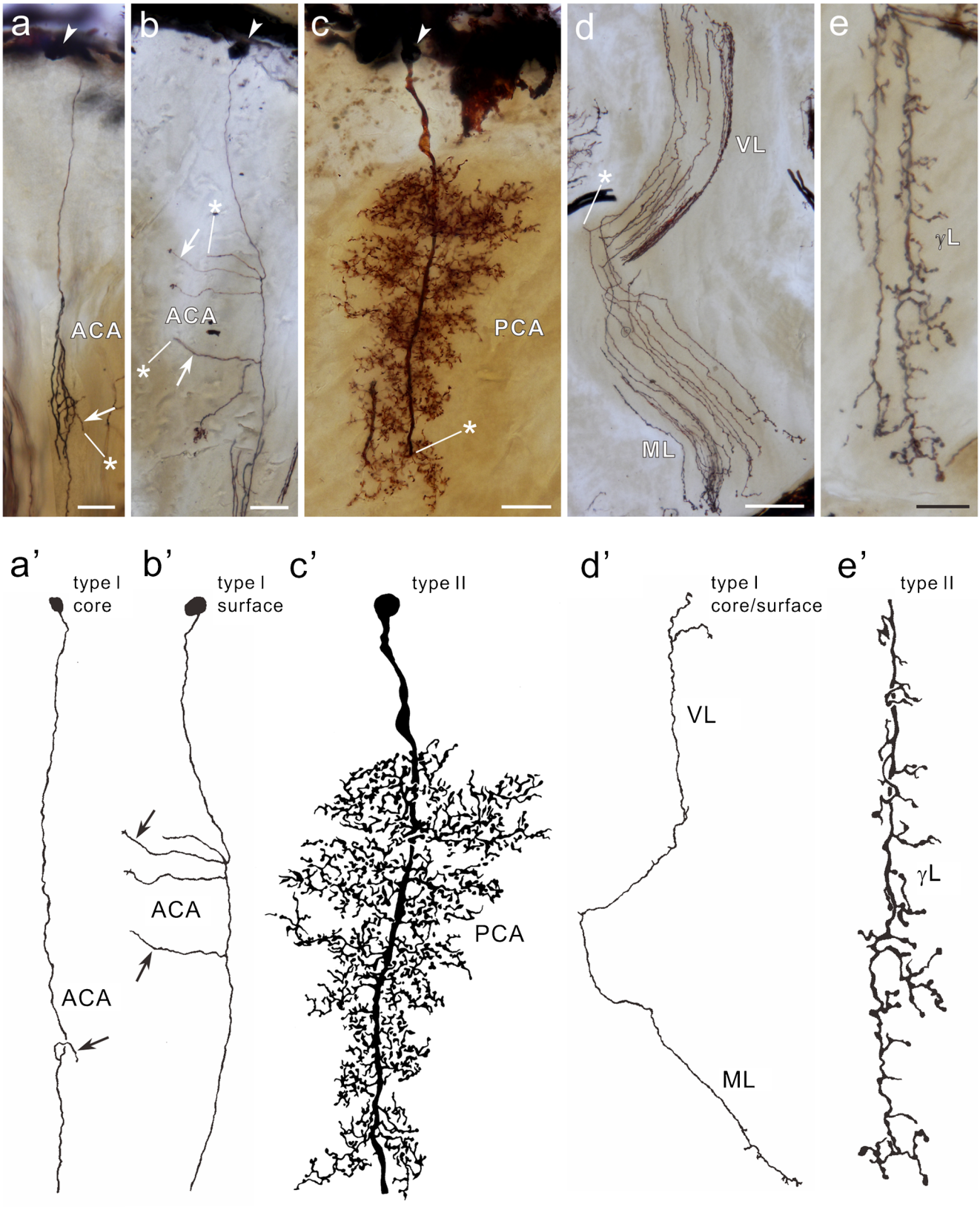
Fig. 1 Gross neuroanatomy of the field cricket's brain. Frontal sections through the mushroom body and the central body (CB). Reduced silver impregnation. **a** The calyx of the mushroom body consists of two compartments, the anteriorly located concave calyx (ACA) and the posteriorly located semicircular calyx (PCA) containing dendritic processes extending from the overlying cell bodies of the Kenyon cells. An unpaired neuropil across the midline, the central body located in the center of the brain, has upper (CBU) and lower (CBL) divisions. **b**,**c** Enlarged images of the anterior calyx and the posterior calyx (dorsal to the top, medial to the right). In the center of the anterior calyx, there is the core of the pedunculus (PED; arrowhead in b) that is probably formed by axons of the newborn Kenyon cells. A bundle of fibers derived from the projection neurons, with dendrites in the antennal lobe (AL), invades the anterior calyx (arrow in c). D, dorsal; LH, lateral horn; P, perikarya; PI, pars intercerebralis. Scale bars (a) 100 μm, (b) 50 μm; b and c have the same scale

widely covering the posterior calyx was impregnated (Fig. 4d). In the pedunculus, many concentric fibers surrounding a bundle of the Kenyon cell axons are visible (a bracket in Fig. 4e).

Tyrosine hydroxylase immunoreactivity in the mushroom body

Tyrosine hydroxylase (TH) immunolabeling has been successfully performed in 9 brains. The TH-immunoreactive fibers widely distribute in the field cricket's brain; however, in the present study, we mainly focused on the mushroom body and the associated antennal lobe in order to characterize the neural substrates underlying associative learning. Every component of the mushroom body was densely invaded by TH-immunoreactive fibers excluding a part of the calyx (Fig. 5a,b), suggesting the important role of dopaminergic neurotransmission in this neuropil. To examine the origin of fibers projecting to different parts of the mushroom body, we completely or partially reconstructed TH-immunoreactive neurons from serially sliced micro-sections so that we could

Fig. 2 Kenyon cells of the mushroom body. Frontal sections through the mushroom body. Golgi preparations (a–d, a'–d': dorsal to the top, medial to the right). **a** Type I core Kenyon cell extending a dendritic branch into the anterior calyx (ACA, arrow). **b** Type I surface Kenyon cell projecting dendritic collaterals with crow-like terminals into the anterior calyx (arrows). **c** Type II Kenyon cell bearing bushy arborizations in the posterior calyx (PCA). Arrowheads in a–c indicate their cell bodies. **d** Axon terminals of the type I core and surface in the vertical lobe (VL) and the medial lobe (ML) bearing sparse collaterals in respective distal parts. **e** Terminals of the type II Kenyon cells in the γ lobe (γL), bearing many short varicose extensions along the axons (for orientation, refer to Fig. 3). **a'**–**e'** Corresponding camera lucida drawings, which show either dendritic or terminal arborizations derived from single neurons in panels a–e. Asterisks in a–d indicate points where neurites are cut by the sectioning plane. Scale bars (a–c, e) 20 μm, (d) 50 μm



identify the four distinct groups of TH-immunoreactive neurons. The number of cell bodies for each group is shown in Table 1. Together, reconstructed TH-immunoreactive neurons cover a part of the anterior calyx, a lateral portion of the

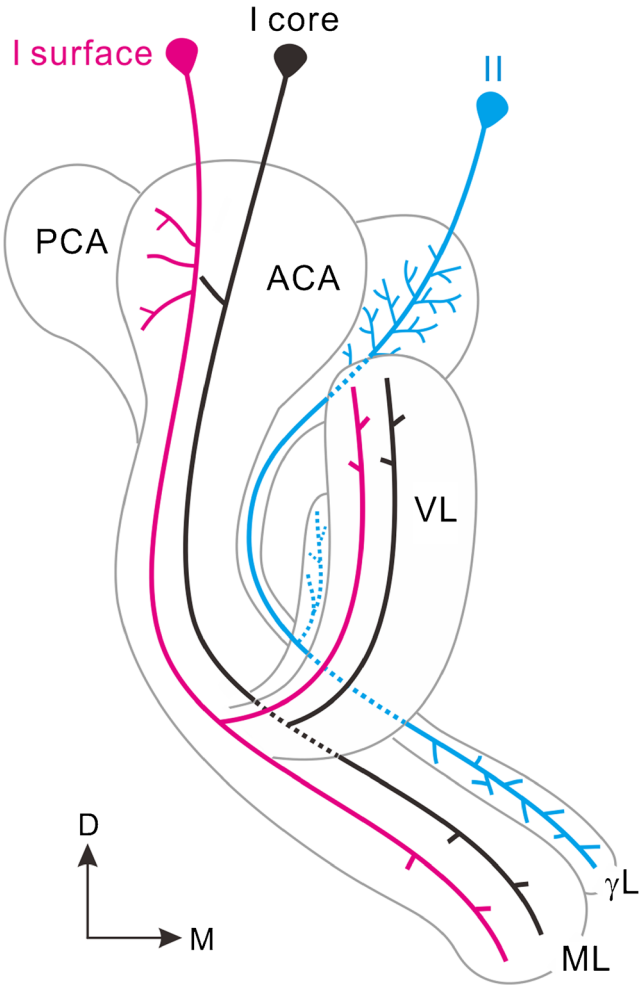


Fig. 3 Diagram of intrinsic Kenyon cells in the cricket's mushroom body. The cricket's mushroom body contains at least three morphologically different types of Kenyon cells: type I core (black), type I surface (magenta) and type II (blue). Type I core and surface may represent age variance of type I. The type I core and surface Kenyon cells project dendritic fibers into the anterior calyx (ACA), and the axons form both the medial lobe (ML) and the vertical lobe (VL), bearing sparse arborizations. The type II forms bushy fine branches in the posterior calyx (PCA) and many varicose arborizations along the axon terminals in the γ lobe (γL). Dotted blue lines in laterally located reduced vertical lobe indicate the plausible fiber trajectory of type II Kenyon cell. D, dorsal; M, medial

posterior calyx, proximal and distal regions of the vertical lobe and a distal area of the medial/γ lobes. The first group of TH-immunoreactive neurons has a cluster of cell bodies in a ventro-lateral region of the anterior calyx (TH-SLP; Fig. 5a,b). These project a bundle of parallel arranged axons antero-ventrally toward the tip of the vertical lobe (Fig. 5c–e), bearing sparse putatively dendritic processes in the superior lateral protocerebral neuropil (Fig. 5a,c). The immunoreactive axons antero-medially bypass the perimeter of the vertical lobe (Fig. 5c–e) and then invade a distal portion of the vertical lobe, through the anterior surface, to bear many fine processes (Fig. 5c,d). In a transverse section of the distal vertical lobe, a granular pattern of labelings is visible predominantly in the outer domain (Fig. 5e). The second group of TH-immunoreactive cell bodies (TH-IP1) is in the inferior medial protocerebrum beneath the medial lobe (Fig. 6a,b). These extend fine branches into distal portions of the medial and γ lobes (Fig. 6a,b). Some short neurites are also visible around the cell bodies. The cell bodies of the third group (TH-IP2) are located in the lateral side of a transition zone between the pedunculus and the lobes (Fig. 6c,d). They extend axons dorsally around the ventral base of the pedunculus and these give rise to fine ramifications in the base of the vertical lobe (Fig. 6c,d). The immunopositive axons extend toward the central body's upper division but their destination was not determined because of the complexity of immunopositive fibers. Besides intense immunoreactivities in the distal portions, the entire part of the medial and γ lobes exhibited a granular pattern of immunolabelings (Fig. 6e). The pedunculus also receives many fine TH-immunopositive fibers of unknown origin, which predominantly reside around the surface (Fig. 6f). The fourth group of TH-immunoreactive neurons is a single multiglomerular projection neuron (TH-MPN; Figs. 7, 8). This was always immunolabeled in each brain hemisphere (Table 1). The cell body is located in the cell body rind lateral to the antennal lobe (arrow in Fig. 7a) and a thick neurite originating from the cell body extends many fibrous processes covering almost all glomeruli of the antennal lobe (Fig. 7a). The main axon runs through a dorsal part of the antennal lobe, enters the medial antennal lobe tract and then extends toward the anterior calyx of the mushroom body (Fig. 7b), in which it sparsely sends off blebby immunopositive fibers (Fig. 7c). Lateral parts of the posterior calyx also receive TH-positive blebby fibers but the middle region lacks immunoreactivity (Fig. 7d). The origin of these immunopositive fibers is not clarified. Running through a neuropil region anterior to the calyx, the axon of the TH-MPN neuron terminates in the dorsal and ventral areas of the lateral horn (Fig. 7e,f). The lateral horn appears to be densely innervated by sets of TH-immunopositive neurons other than the TH-MPN neuron. The principal fiber trajectory and cell body locations of the four distinct classes of TH-immunoreactive neurons are summarized in Fig. 9.

Discussion

In order to identify putative dopaminergic neurons, we employed an antiserum against tyrosine hydroxylase (TH), a rate-limiting enzyme in dopamine biosynthesis (Blenau and Baumann 2001; Roeder 2002). It has been demonstrated that TH is expressed in most putative dopaminergic neurons in the fruit fly, *Drosophila melanogaster* (Friggi-Grelin et al. 2003) and that dopamine- and TH-immunoreactivities are colocalized in several groups of brain neurons in the

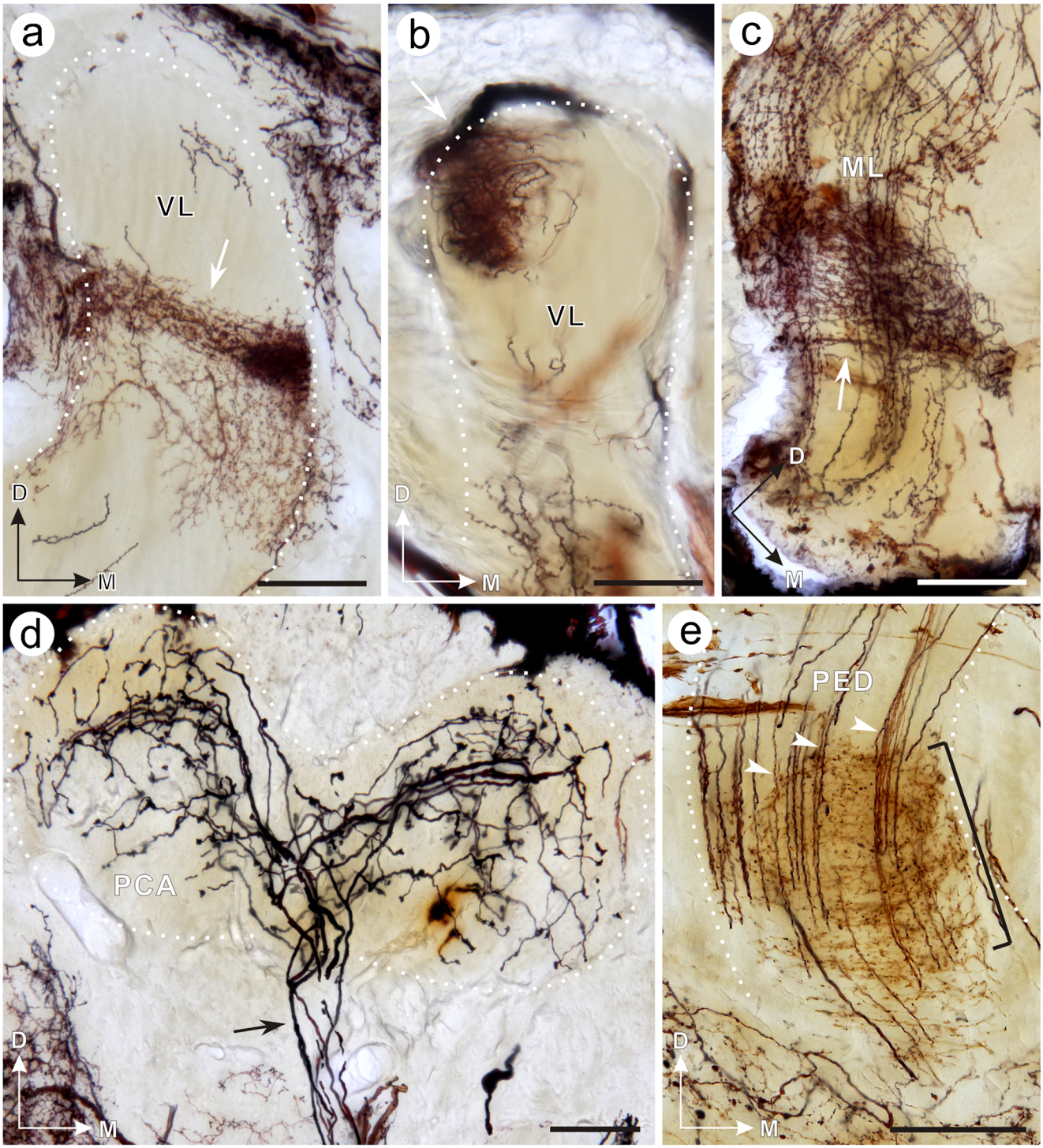
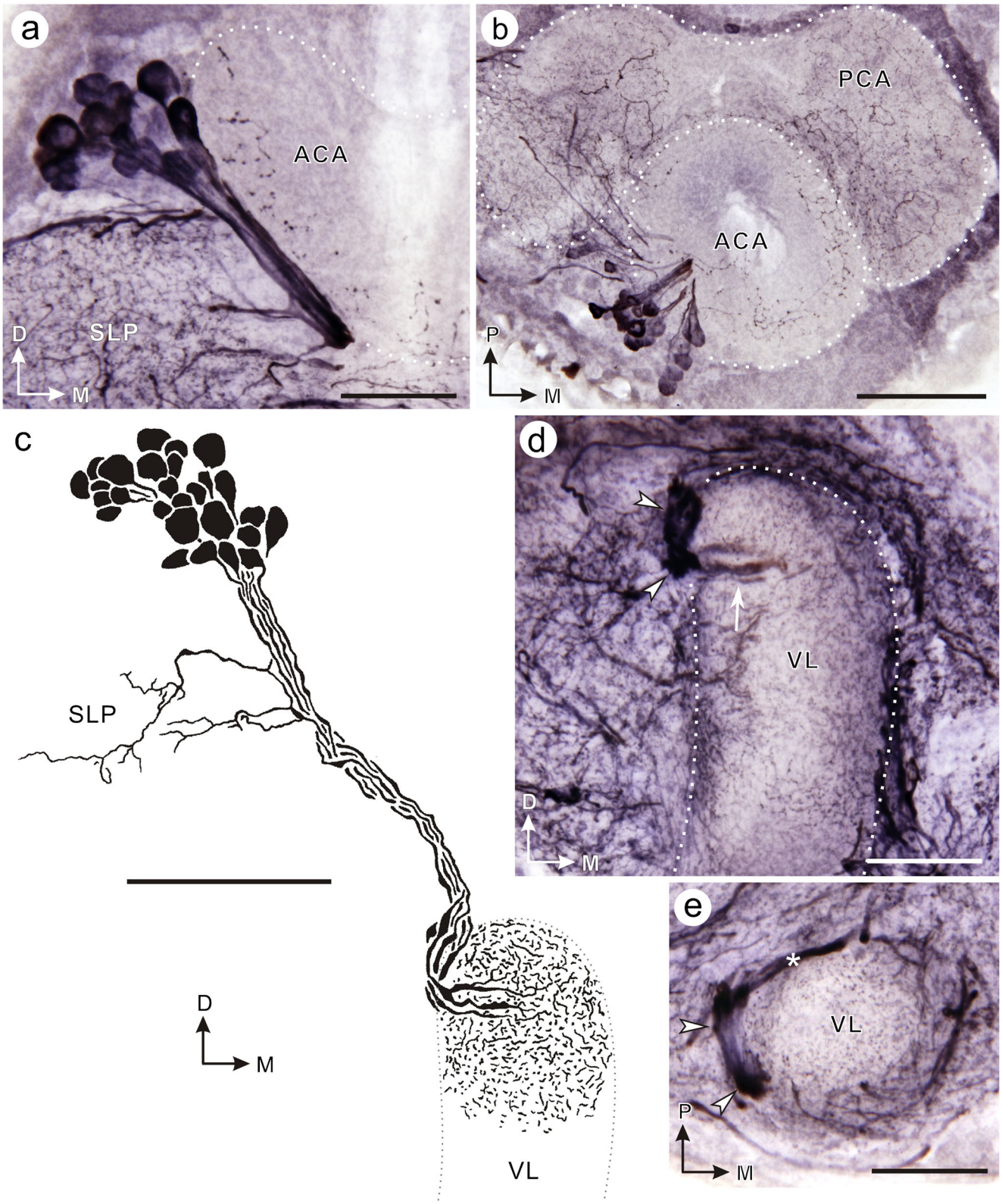


Fig. 4 Extrinsic fibers projecting to the mushroom body. Frontal sections through the vertical lobe (VL; a, b), medial lobe (ML; c), posterior calyx (PCA; d) and pedunculus (PED; e) of the mushroom body. Golgi preparations. **a** Extrinsic fibers invading the vertical lobe perpendicularly to the axis of Kenyon cell axons (arrow). **b** A thick fiber invading the lateral tip of the vertical lobe, bearing fine ramifications (arrow). **c** Fibers running across the Kenyon cell axons in the medial lobe (arrow). **d** Axons entering the posterior calyx (arrow) ramify there to extend blebby fibers widely. **e** Concentric fibers in the pedunculus (bracket), surrounding the axons of Kenyon cells (arrowheads). D, dorsal; M, medial. Scale bars 50 μm

American cockroach, *Periplaneta americana* (Hamanaka et al. 2016). These validate the use of the anti-TH antiserum as a marker for dopaminergic neurons. In the field cricket, the distribution patterns of TH/dopamine-immunoreactive neurons have been comprehensively examined in the subesophageal zone and the thoracic and abdominal ganglia



but not much in the brain (Hörner et al. 1995). Hörner et al. (1995) described only the number of immunopositive cells in the brain but they did not provide information of their locations and axon trajectories, which are informative in evaluating the sites of dopamine action for each class of dopaminergic neuron. In the present study, we completely or partially reconstructed the morphologies of four distinct classes of putative dopaminergic neurons that project to different

◀ **Fig. 5** Tyrosine hydroxylase-immunoreactive neurons projecting to the vertical lobe. Frontal sections through the anterior calyx (ACA; a) and the vertical lobe (VL; d). Horizontal sections through the calyx (b) and distal vertical lobe (e). **a** A dozen tyrosine hydroxylase-immunoreactive cell bodies located in the vicinity of the anterior calyx (TH-SLP neurons) extend putatively dendritic fibers in the superior lateral protocerebral neuropil (SLP). Central and dorsal regions of the anterior calyx are lacking immunopositive fibers. **b** Cell bodies of the TH-SLP neurons in the antero-lateral base of the anterior calyx. In the anterior calyx, more immunoreactive fibers are visible in the anterior half. In the posterior calyx (PCA), the lateral regions receive immunopositive fibers while the medial portion lacks such fibers. **c** Frontal reconstruction of the TH-SLP neurons. They extend putative dendritic processes in the superior lateral protocerebral neuropil and densely innervate the distal vertical lobe. **d**,**e** Axons extending to the distal portion of the vertical lobe (arrowheads), derived from the TH-SLP neurons. The immunoreactive axons briefly run antero-medially (arrowheads in d, e) and invade the distal vertical lobe from the anterior surface (arrow in d) to bear many ramifications (c). Asterisk (in e) indicates axons that are not attributed to the TH-SLP neurons. D, dorsal; M, medial; P, posterior. Scale bars (a, d, e) 50 μm, (b, c) 100 μm

portions of the mushroom body, as a first step to identify dopaminergic neurons crucial for aversive memory formation in the field cricket, *Gryllus bimaculatus*.

The mushroom body

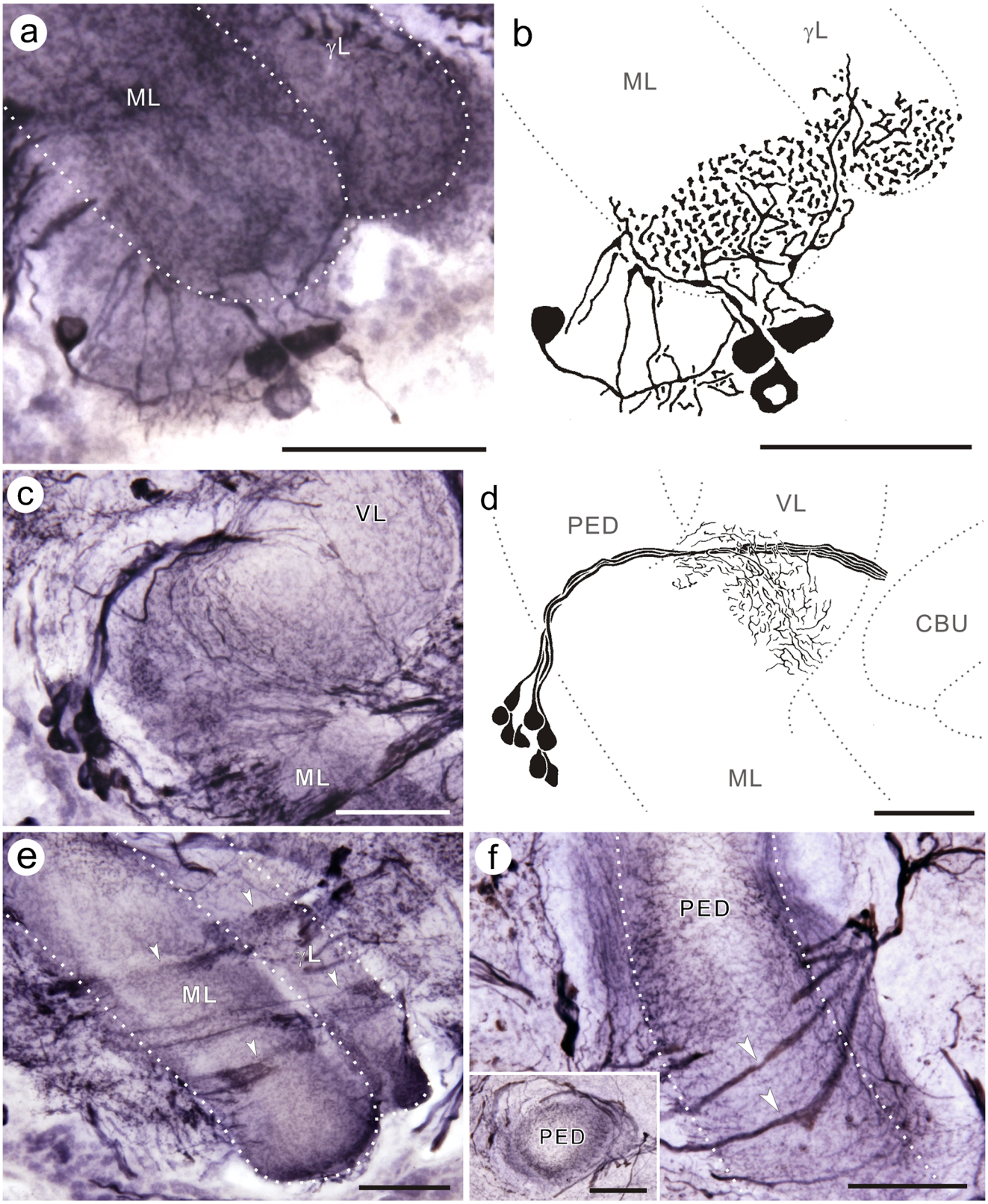
The mushroom bodies in insects are a higher integration center for various modalities of sensory stimuli and for associative learning (Heisenberg 2003; Giurfa 2007; Mizunami et al. 2013, 2015; Waddell 2013; Owald and Waddell 2015; Cognigni et al. 2018; Hige 2018). This neuropil consists of intrinsic Kenyon cells and extrinsic afferent and efferent fibers. The extrinsic fibers connect the mushroom body to the other brain centers. Despite their importance, reports in the field cricket on the morphologies of the Kenyon cells and extrinsic neurons at single-cell level have been few (Schürmann et al. 2008; Schürmann 2016). In the present study, we revealed two distinct classes of Kenyon cells (types I and II) by Golgi impregnation, the former was further divided into two subtypes (types I core and I surface). Corresponding classes exist in the house cricket whose mushroom body consists of 50,000 Kenyon cells per hemisphere (Schürmann 1973). This number is 20 times larger than that of the fruit fly (2500 per hemisphere) (Hinke 1961; Balling et al. 1987) while smaller than that of the honey bee (170,000 per hemisphere) (Mobbs 1982) and the cockroach (200,000 per hemisphere) (Neder 1959). The volume of the mushroom body in the two cricket species appears to be approximately equal and thus, the mushroom body of the field cricket probably houses a similar number of Kenyon cells to that of the house cricket. The well-developed but seemingly less complex organization of the mushroom body is inconsistent with the high capability of various forms of learning of this species (Mizunami and Matsumoto 2010; Mizunami et al. 2013, 2015) but ideal to dissect the neural bases that underlie various forms of learning, such as olfactory, visual, spatial, contextual and social learning. In addition to the Kenyon cells, Golgi impregnation revealed five different types of extrinsic fibers, which invade distinct compartments in the calyx, the pedunculus and the lobes. Such a compartmentalization probably reflects functional specification within these substructures as reported in the fruit fly (Owald and Waddell 2015; Cognigni et al. 2018; Hige 2018).

In the field cricket *G. bimaculatus*, unlike the fruit fly, the aversive and appetitive reinforcement are mediated in parallel by dopamine and octopamine, respectively (Mizunami and Matsumoto 2010; Mizunami et al. 2013, 2015; Awata et al. 2015, 2016). Furthermore, it has been demonstrated that a dopamine receptor, Dop1, one among four dopamine receptors (Watanabe et al. 2013) is crucial for aversive memory formation (Awata et al. 2015, 2016), which is predominantly expressed in a subset of the Kenyon cells surmounting the anterior calyx (Hamada et al. 2009). According to the cell body location, the Dop1-expressing Kenyon cells appear to be type I surface Kenyon cells identified in the present study (Figs. 2b, b', 3), which preferentially receive olfactory inputs through the olfactory projection neurons (Frambach and Schürmann 2004). Thus, type I surface Kenyon cells seem to mediate the conditioned stimulus (CS) in olfactory associative learning. Another type II Kenyon cell extending dendrites into the posterior calyx most likely receives chemosensory inputs from the maxillary palps via other classes of projection neurons (Frambach and Schürmann 2004). Type I core Kenyon cells have only a short extension in the anterior calyx but the axon trajectory is indistinguishable from that of the

Table 1 Location of the cell bodies of tyrosine hydroxylase-immunoreactive neurons projecting to the mushroom body in the field cricket, *Gryllus bimaculatus*

Cell group	Number of cell bodies per hemisphere[a]	Location of cell bodies	Projections
TH-SLP	28	Ventro-lateral to the anterior calyx	Superior lateral protocerebral neuropil and distal vertical lobe
TH-IP1	10	Inferior medial protocerebrum	Distal part of the medial lobe and γ lobe
TH-IP2	27	Inferior protocerebrum	Proximal part of the vertical lobe and possibly the central body upper division?
TH-MPN	1	Deutocerebrum lateral to the antennal lobe	Antennal lobe, anterior calyx, lateral horn

[a] Maximum number of tyrosine hydroxylase-immunoreactive cell bodies is shown



type I surface cells. The type I core cells seem to be an immature form of type I surface Kenyon cells. In the house cricket, the small Kenyon cells (corresponding to the type I surface in the field cricket) are born from a group of neuroblasts at the center of the anterior calyx even in adulthood and the newborn Kenyon cells are pushed to the periphery as they mature

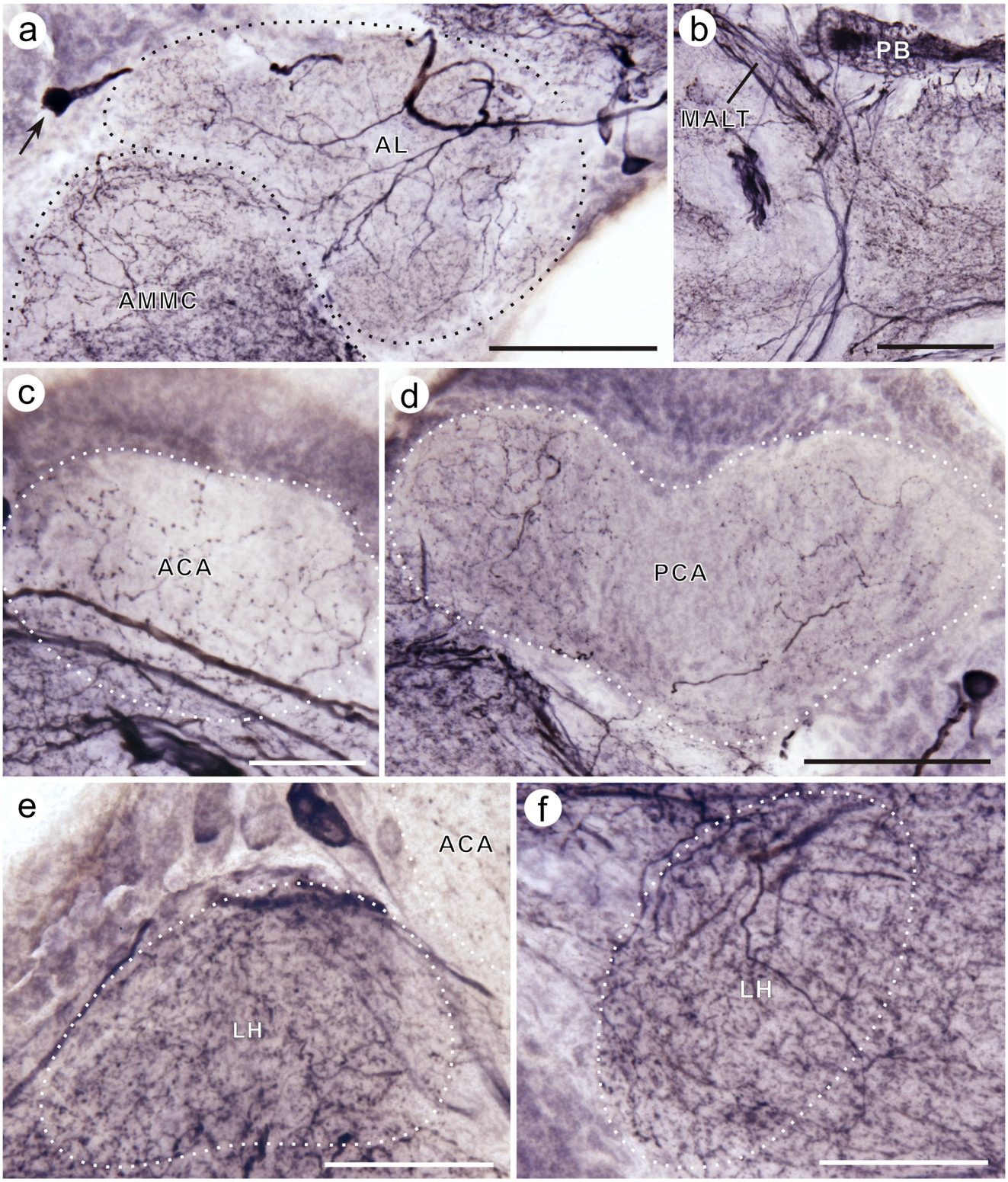
◀ **Fig. 6** Tyrosine hydroxylase immunoreactivity in the pedunculus and lobes of the mushroom body. Frontal sections through the medial lobe (ML) and γ lobe (γL; a, e), the junction between the pedunculus (PED) and the lobes (c) and the pedunculus (f). Dorsal to the top and medial to the right. **a** Tyrosine hydroxylase-immunoreactive neurons extending the fibers to the medial and γ lobes (TH-IP1). Immunopositive fibers invade predominantly the distal portions. **b** Camera lucida drawing of the TH-IP1 neurons in panel a. **c** Tyrosine hydroxylase-immunoreactive neurons extending axons toward the central body upper division (CBU, TH-IP2). About 8 cell bodies are visible in this section. They give rise to fine branches in the base of the vertical lobe (VL). **d** Camera lucida drawing of TH-IP2 neurons in panel c. **e** Medial and γ lobes exhibiting a granular pattern of immunolabelings through the structures. A few bundles of immunopositive fibers intersect the Kenyon cell axons in both lobes (arrowheads). **f** The pedunculus receives fine immunopositive fibers, more densely in the outer region (also see the horizontally sliced pedunculus in the inset). Some thick axons bypassing the pedunculus are also visible (arrowheads). Scale bars 50 μm

(Malaterre et al. 2002). Alternatively, the type I core might belong to a functionally distinct class.

In the present study, we identified four distinct classes of putative dopaminergic neurons as summarized in Fig. 9: three classes projecting to the different compartments in the lobes (TH-SLP, TH-IP1 and TH-IP2) and the fourth class, a multiglomerular projection neuron invading the anterior calyx (TH-MPN). The TH-SLP neurons possess putatively dendritic fibers in the superior lateral protocerebral neuropil and project their axons into the distal vertical lobe. This is the most promising candidate for the one that conveys aversive signals (unconditioned stimulus, US) into the lobes to modulate synaptic efficiency from the Kenyon cells (representing the CS) to the mushroom body's output neurons (possibly commanding avoidance behavior) in aversive learning. In the fruit fly, three distinct groups of dopaminergic PPL1 neurons with cell bodies ventro-lateral to the calyx (designated as MB-MP1, MB-MV1 and MB-V1) convey aversive reinforcement signals into the distinct mushroom body's compartments in olfactory and visual (color) aversive memory formation (Claridge-Chang et al. 2009; Aso et al. 2010, 2012; Vogt et al. 2014). The MB-MP1 invades the γ1 lobe and the core of the distal pedunculus, the MB-MV1, the γ2 and α'1 lobes and MB-V1 α'2 and α2 lobes, with all types bearing the putatively dendritic arborizations in the anterior inferior medial protocerebral neuropil (Aso et al. 2014). The MB-MP1 is also shown to mediate hunger-dependent motivational control over appetitive memory expression (Cognigni et al. 2018). The TH-SLP neurons resemble these PPL1 neurons, according to their cell body location and axon trajectory. Anatomically homologous dopamine/TH-immunoreactive neurons are also identified in the cockroach (DCa1) (Hamanaka et al. 2016) and the honey bee (a subset of C_3 neurons) (Schäfer and Rehder 1989; Tedjakumala et al. 2017). As in the field cricket and the fruit fly, dopaminergic neurons play important roles in aversive olfactory and visual learning in the honey bee (Vergoz et al. 2007). The TH-SLP neurons in the field cricket and their counterparts in the cockroach and honey bee may play a key role in the association between the CS and the noxious US. Another candidate providing aversive reinforcement signals to the mushroom body's lobe is the TH-IP2 neurons that extend fibers to a basal part of the vertical lobe. Whether the TH-SLP and/or TH-IP2 neurons are involved in aversive memory formation in the field cricket remains to be studied, however. The third candidate is the TH-IP1 neurons projecting to distal regions of the medial and γ lobes. In the fruit fly, a set of dopaminergic PAM neurons that correspond anatomically to the TH-IP1 neurons mediates appetitive reinforcement signals in olfactory and visual learning (Burke et al. 2012; Liu et al. 2012; Vogt et al. 2014). However, it is unlikely that the TH-IP1 neurons are involved in appetitive memory formation since a reward signal is unambiguously conveyed by octopaminergic neurons in the field cricket (Mizunami et al. 2013, 2015). Further work will be needed to show whether the TH-IP1 neurons are involved in aversive learning or not. The fourth type is a TH-MPN neuron. This is the second report implying that a subset of projection neurons utilizes dopamine as a neurotransmitter, following our report in the cockroach (Hamanaka et al. 2016). The TH-MPN neuron resembles a multiglomerular neuron in the fruit fly classified as AL-mPN4 (Tanaka et al. 2012). In the fruit fly, a type of projection neuron exhibits synaptic activity in its dendrites in the glomerulus of the antennal lobe in response to the US of mild electrical shock but not to a CS of 3-octanol before conditioning but they exhibit strong synaptic activation in response to the CS alone shortly after pairing the CS with the US (Yu et al. 2004). Altered odor representation after associative learning is also reported in the antennal lobe of the honey bee (Denker et al. 2010). These suggest that a short-term memory trace is formed even in the primary olfactory center of the antennal lobe. In response to concomitant or sequential perception of an odor and a noxious sensory input, the TH-MPN neurons in the field cricket may modify the synaptic connections between either the olfactory receptor neurons or their local interneurons and the conventional uniglomerular projection neurons. If this occurs, then the application of a noxious sensory cue may accompany the dendritic release of dopamine from TH-MPN neurons in the field cricket. This possibility needs to be investigated further.

Comparative aspects of dopaminergic neurons projecting to the mushroom body

Putative dopaminergic neurons projecting to the mushroom body have been demonstrated in the cockroach



(Hamanaka et al. 2016), flies (Nässel and Elekes 1992; Mao and Davis 2009; Aso et al. 2014), honey bee (Schäfer and Rehder 1989; Tedjakumala et al. 2017), locust (Wendt and Homberg 1992) and cricket (present study). Some of these neurons are very well-conserved between species although some minor differences also exist, suggesting conservation and species-specific variations of their roles in the mushroom body. The most striking similarity is seen in neurons projecting to the lobes. According to the location of their cell bodies and axon

◀ **Fig. 7** Tyrosine hydroxylase immunoreactivity in the protocerebrum and deutocerebrum. Frontal sections through the antennal lobe (AL; a), the medial antennal lobe tract (MALT; b), the anterior calyx (ACA; c), the posterior calyx (PCA; d) and the lateral horn (LH; e, f). Dorsal to the top and medial to the right. **a** Antennal lobe is widely covered by putative dendritic fibers of a single projection neuron (TH-MPN). An arrow indicates the cell body, from which a thick neurite extends and enters into the medial antennal lobe tract (b), to project toward the anterior calyx. **b** Medial antennal lobe tract and the protocerebral bridge (PB) containing intensively immunolabeled processes. **c** Anterior calyx receiving sparse beaded fibers of the TH-MPN neurons, more fibers in the ventral portion. **d** Posterior calyx receiving TH-immunoreactive processes. The fibers invade the lateral parts but not the center. **e**,**f** Dorsal (e) and ventral (f) regions of the lateral horn receiving many immunopositive fibers, some of which are attributed to the TH-MPN neuron. For projection pattern of the TH-MPN neuron, also see Fig. 8. AMMC, antennal mechanosensory and motor center. Scale bars (a, b, d) 100 μm, (c, e, f) 50 μm

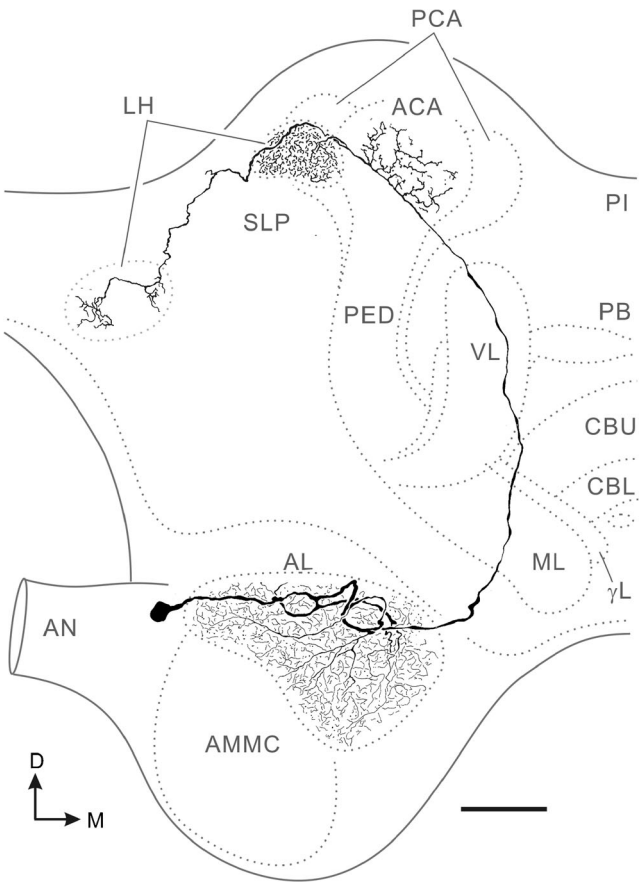


Fig. 8 Frontal reconstruction of a tyrosine hydroxylase-immunoreactive multi-glomerular projection neuron (TH-MPN). This neuron, the cell body of which is located lateral to the antennal lobe (AL), extends dendritic branches in the antennal lobe, bearing many fine fibers over most of the glomeruli. The axon runs through the medial antennal lobe tract to project to the anterior calyx (ACA) of the mushroom body and dorsal and ventral parts of the lateral horn (LH). AMMC, antennal mechanosensory and motor center; AN, antennal nerve; CBL, central body lower division; CBU, central body upper division; D, dorsal; M, medial; ML, medial lobe; VL, vertical lobe; PB, protocerebral bridge; PCA, posterior calyx; PED, pedunculus; PI, pars intercerebralis; γL, γ lobe. Scale bar 100 μm

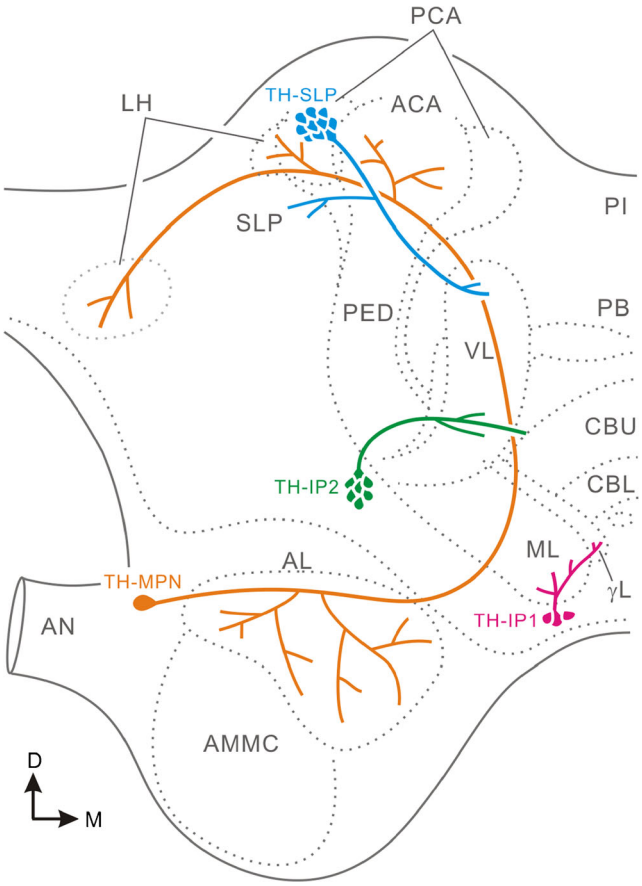


Fig. 9 A diagram depicting cell body locations and principal fiber trajectories of four distinct classes of tyrosine hydroxylase-immunoreactive neurons projecting to the mushroom body in the field cricket, *Gryllus bimaculatus*. All abbreviations for neuropil structures are as in Fig. 8

projection pattern, the TH-SLP neurons in the field cricket display close resemblance to DCa1 neurons in the cockroach (Hamanaka et al. 2016), a subset of C_3 neurons in the honey bee (Schäfer and Rehder 1989; Tedjakumala et al. 2017) and PPL1 neurons in flies (Nässel and Elekes 1992; Aso et al. 2014). These have cell bodies in the cell body rind ventro-lateral to the calyx and extend the fibers to the vertical and/or medial lobes. Other conserved cell clusters are in the inferior protocerebrum. In the field cricket, a cluster of TH-immunoreactive cells (TH-IP1 neurons) are located beneath the medial lobe and they project their branches into the distal portions of the medial and γ lobes. Anatomically corresponding neurons are C_1 neurons in the honey bee (Schäfer and Rehder 1989; Tedjakumala et al. 2017), DIP1 neurons in the cockroach (Hamanaka et al. 2016), DIP1 neurons in the locust (Wendt and Homberg 1992) and PAM neurons in flies (Nässel and Elekes 1992; Aso et al. 2014). Another cell cluster (TH-IP2) in the field cricket may be homologous to C_2 neurons in the honey bee (Schäfer and Rehder 1989; Tedjakumala et al. 2017), DIP2 neurons in the

cockroach (Hamanaka et al. 2016) and DIP2 neurons in the locust (Wendt and Homberg 1992). Moreover, we identified a single multiglomerular projection neuron (TH-MPN) per brain hemisphere. This sends olfactory information into the anterior calyx and the lateral horn. Such a putatively dopaminergic neuron has been reported in the cockroach as well (Hamanaka et al. 2016).

Plausible dopamine neurons mediating aversive prediction error signals

Our recent study demonstrated that the prediction error theory, which underlies associative learning in mammals (Rescorla and Wagner 1972; Schultz 2006, 2013), is applicable to the field cricket (Terao et al. 2015; Terao and Mizunami 2017). In this theory, a discrepancy or an error between the actual US and the predicted US from the CS (prediction error) determines whether learning occurs or not. In the field cricket, the appetitive and aversive prediction error are suggested to be mediated by octopaminergic and dopaminergic neurons innervating the lobes of the mushroom body, according to the model proposed in this cricket (Terao et al. 2015; Terao and Mizunami 2017). The roles of the lobes in associating the CS with the US have been well-characterized in the fruit fly (Owald and Waddell 2015; Cognigni et al. 2018; Hige 2018). The present study identified three types of putatively dopaminergic neurons projecting to the mushroom body lobes (TH-SLP, TH-IP1 and TH-IP2). It is an intriguing question whether either of these is responsible for mediating the prediction error signal for aversive memory formation.

Neural computation of the prediction error remains unclear in any species. Since neurons in the olfactory system in the cricket are suitable for intracellular recording (Schildberger 1984), it is awaited to address whether or how the immunohistochemically identified dopamine neurons change the activity patterns of the mushroom body's output neurons during the process of conditioning. Moreover, analysis using optogenetics may also be possible in the near future because the knock-in technique has already been established in the field cricket using a genome-editing method (Watanabe et al. 2017). Due to these technical advances combined with its electrophysiological accessibility, the field cricket proves to be a valuable model to study the neural mechanisms underlying associative learning, including that of prediction error computation.

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