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Tritocerebral tract input to the insect mushroom bodies

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ABSTRACT

Insect mushroom bodies, best known for their role in olfactory processing, also receive sensory input from other modalities. In crickets and grasshoppers, a tritocerebral tract containing afferents from palp mechanosensory and gustatory centers innervates the accessory calyx. The accessory calyx is uniquely composed of Class III Kenyon cells, and was shown by immunohistochemistry to be present sporadically across several insect orders. Neuronal tracers applied to the source of tritocerebral tract axons in several species of insects demonstrated that tritocerebral tract innervation of the mushroom bodies targeted the accessory calyx when present, the primary calyces when an accessory calyx was not present, or both. These results suggest that tritocerebral tract input to the mushroom bodies is likely ubiquitous, reflecting the importance of gustation for insect behavior. The scattered phylogenetic distribution of Class III Kenyon cells is also proposed to represent an example of generative homology, in which the developmental program for forming a structure is retained in all members of a lineage, but the program is not "run" in all branches.

1. Introduction

The insect mushroom bodies are higher brain centers best studied for their roles in sensory integration and in certain types of learning and memory (cricket, Schildberger, 1984; cockroach, Li and Strausfeld, 1997, 1999; honey bee, Menzel, 2001; fruit fly, Roman and Davis, 2001; Heisenberg, 2003). Both types of studies have identified the mushroom bodies as centers of particular importance for olfactory processing and discrimination, a finding that has been well-supported by physiological studies (locust, Perez-Orive et al., 2002; honey bee, Szyszka et al., 2005). Dedicated sensory input neuropils of the mushroom bodies, called calyces, receive massive input from primary olfactory centers called the antennal lobes via one or more antennocerebral tracts (ACTs) (house fly, Strausfeld, 1976; cockroach, Strausfeld and Li, 1999a; honey bee, Kirschner et al., 2006). A strong case can also be made for the adaptive significance of this neural pathway, as olfactory cues guide key aspects of insect behavior such as the location of food, mates and oviposition sites (reviewed in Christensen and Hildebrand, 1987; van der Goes van Naters and Carlson, 2006). Higher processing functions provided by the mushroom bodies, for example learning the context of particular cues or associating an array of cues in time and space, may be an important component of these olfactory-guided behaviors (Liu et al., 1999; Liu and Davis, 2006).

While olfactory inputs to the calyx are widespread among the insects and are therefore likely to have been present in a common ancestor, the influence of species-specific behavioral ecologies on calyx innervation becomes apparent when comparisons are made among insects with unique evolutionary specializations. For example, diminutive antennae and the reduction or loss of the entire antennal lobe – inner ACT – mushroom body calyx pathway is observed in notonectid Heteroptera, which have adopted a fully aquatic lifestyle devoid of airborne olfactory cues (Strausfeld et al., 1998). Another example occurs in the Hymenoptera, particularly in bees and wasps, in which the calyces receive visual input from the medulla and lobula of the optic lobes (Gronenberg, 2001; Gronenberg and Hölldobler, 1999). This is likely related to the important role that vision plays in learning the location of food sources and nest sites and even in identifying conspecifics in these insects (Menzel et al., 2005; Tibbetts, 2002). The association between mushroom body afferents and behavior is further supported by the finding that ant species that rely less on vision for the above tasks have a consequent reduction in visual input to the calyces (Gronenberg and Hölldobler, 1999).

Another important sensory modality for insects is contact chemoreception or gustation, which is used to determine the suitability of food sources and oviposition sites on contact (Justus and Mitchell, 1996; Chapman, 2003; Romani et al., 2005; van der Goes van Naters and Carlson, 2006). Mechanosensation is also of importance as it allows insects to gather information about texture that may be used to discriminate and learn about different foods (Kevan and Lane, 1985; Goyret and Raguso, 2006). Gustatory receptors are distributed in species-specific patterns across the

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antennae and mouthparts and usually occupy sensilla that also contain mechanosensory receptors (Mitchell et al., 1999). These gustatory sensilla characteristically possess a single tip pore and a moveable base (Staudacher et al., 2005). In holometabolous insects, gustatory sensilla are typically found on parts of the maxillae and their palps, or on modified structures of the labium (such as the long tongue-like glossa in bees and the sponge-like labellum of flies) (Mitchell et al., 1999), and on the antennae (Haupt, 2004; Jorgensen et al., 2006). However gustatory sensilla are arranged peripherally, the receptor neurons of holometabolous species project to the subesophageal ganglion (SEG) and in some cases the tritocerebrum (TC), both of which appear to function as primary gustatory and mechanosensory centers in the Holometabola (Ignell and Hansson, 2005; Jorgensen et al., 2006; Mitchell et al., 1999).

Ascending pathways from centers receiving input from gustatory sensilla in the SEG and TC to the mushroom bodies have been described in just one holometabolous insect, the honey bee (Schröter and Menzel, 2003). Neurons comprising the subesophageal-calycal tract (SCT) have cell bodies residing along the lateral edge of the SEG-TC junction, and dendritic fields that overlap with the termini of sensory receptor axons originating from the mouthparts (Rehder, 1989). The SCT runs first along the medial deutocerebrum and protocerebrum, where it appears nearly indistinguishable from the inner ACT (iACT) carrying olfactory projection neuron axons from the antennal lobes. At approximately the level of the mushroom body medial lobe, however, the SCT cuts away from the iACT, passes ventral to the calyces, and provides collaterals to mushroom body intrinsic neurons comprising distinct calycal subcompartments before terminating in the lateral protocerebrum.

As Schröter and Menzel (2003) point out, a similar ascending pathway is well known from another group of insects, the hemimetabolous Orthoptera (crickets and grasshoppers). Comparative studies by Jawlowski (1954) and Weiss (1981) identified what was termed the tritocerebral tract (TT) emerging from a glomerular neuropil at the tritocerebral-deutocerebral border (the lobus glomerulatus or LG). The TT follows the exact trajectory of the SCT through the protocerebrum, but unlike in the honey bee where SCT collaterals target subcompartments of the primary calyces (that also receive visual and olfactory input), TT axons in the Orthoptera provide collaterals to a physically separate mushroom body neuropil termed the accessory calyx. More recent studies have verified this finding using neuronal tracing and immunohistochemistry techniques (Frambach and Schürmann, 2004; Homberg et al., 2004).

Of greater interest to the present account are the cell populations in the mushroom bodies that are targeted by TT/SCT axons. Variation is already evident in the above comparison between orthopterans and bees, as the TT projects to a separate accessory calyx in the former, but to subcompartments of the primary calyces in the latter. Developmental analyses of accessory calyces in two species, the cricket *Acheta domestica* and the cockroach *Periplaneta americana*, reveal that the component intrinsic neurons (termed Class III Kenyon cells by Farris and Strausfeld (2003) are the first-born during embryonic development (Malaterre et al., 2002; Farris and Strausfeld, 2003). Aside from forming an accessory calyx, Class III Kenyon cells characteristically produce physically separate, delay line-like tracts that run alongside or wrap around the pedunculus and lobes (Malaterre et al., 2002; Farris and Strausfeld, 2003; Sjöholm et al., 2005).

In the Orthoptera where they are best studied, Class III Kenyon cells receive massive input from the LG via the TT (Frambach and Schürmann, 2004). In the honey bee, in which Class III Kenyon cells have not been observed in the adult (Farris et al., 2004), TT axons instead provide collaterals to a subcompartment of the primary calyx, which is composed of Class II and Class I Kenyon cells (Schröter and Menzel, 2003; Strausfeld, 2002). This suggests that when present, Class III Kenyon cells serve a specific function in gustatory processing, but that other cell populations in the mushroom bodies can receive this input modality as well.

The goal of the current study is to compare the distribution of TT/SCT inputs to the mushroom bodies across representatives of several insect orders. Using immunostaining and neuronal tract tracing, TT/SCT inputs are found to be ubiquitous but diverse, while accessory calyces and their constituent Class III Kenyon cells have a less uniform distribution.

2. Materials and methods

2.1. Insects

Thermobia domestica (Lepismatidae: Zygentoma) were reared in a laboratory incubator at 35 °C on a 24 h dark cycle. They were fed Meow Mix® cat food *ad libitum* and moisture was provided by pans of tap water placed at the bottom of the incubator. *Periplaneta americana* (Blattidae: Dictyoptera) and *Acheta domesticus* (Gryllidae: Orthoptera) were reared in an incubator at 28 °C on a 12:12 light:dark cycle and fed cat food. Water was provided by soaking paper towels contained within plastic cups, that were replaced every week. *Oncopeltus fasciatus* (Lygaeidae: Hemiptera) were reared in an incubator at 28 °C on a 12:12 light:dark cycle and provided with raw, shelled sunflower seeds for food. Water was provided in a closed cup with a central wick. *Tetrix* sp. (Tetrigidae: Orthoptera), *Ceuthophilus* sp. (Raphidophoridae: Orthoptera), *Forficula auricularia* (Forficulidae: Dermaptera), *Onthophagus hecate* and *Maladera castanea* (Scarabaeidae: Coleoptera) were captured live in the Morgantown, WV area and prepared within 24 h for neuronal filling. *Tachycines asynamorous* (Raphidophoridae: Orthoptera) were collected in Chincoteague, VA and prepared within 48 h for immunostaining.

The following sample sizes were used for this study: Cason's staining *Thermobia* first instar $n = 8$; Cason's staining *Thermobia* adult $n = 6$; DC0/phalloidin staining *Oncopeltus* $n = 6$; DC0 staining/deutocerebrum fills *Forficula* $n = 2$; DC0/phalloidin staining *Acheta* $n = 2$; DC0/phalloidin staining *Tachycines* $n = 2$; DC0/phalloidin staining *Periplaneta* $n = 2$; antennal nerve fills *Thermobia* $n = 10$; deutocerebrum fills *Thermobia* $n = 5$; deutocerebrum fills *Periplaneta* $n = 5$; deutocerebral fills *Tetrix* $n = 2$; deutocerebral fills *Ceuthophilus* $n = 2$; deutocerebral fills *Onthophagus* $n = 2$; deutocerebral fills *Maladera* $n = 5$.

2.2. Fluorescent dye tracing of neuronal trajectories

Prior to TT/SCT filling with fluorescent dextran, insects were anesthetized on ice until movement ceased. Heads were then removed and the brain rapidly dissected under O'Shea–Adams saline (O'Shea and Adams, 1981). All fills were performed with Texas Red conjugated 3000 MW dextran (Molecular Probes, Inc. (Invitrogen), Eugene, OR). Two methods were used to introduce the dye into the brain. In the first, highly concentrated liquid dye (≥5% in distilled water) was loaded into a broken-ended glass electrode, of which the blunt end was attached to a tuberculin syringe via a piece of rubber tubing. The point of the electrode was placed in the desired fill site and the syringe plunger depressed, pressure-injecting the dye into the brain. The second method used a similarly broken electrode, but rather than loading with liquid dye, semi-hardened dye on the sides of an Eppendorf tube was simply used to coat the tip of the electrode. The dye was then directly pushed into the brain as the electrode was inserted. Whichever method employed, the dye was either introduced into the LG (*Ceuthophilus*, *Tetrix*, *Periplaneta*, *Forficula*), or the point in the medial antennal lobe at which the TT/SCT and the ACT are confluent (*Thermobia*, *Onthophagus*, *Maladera*). All fill sites produced labeling of cells with trajectories in